

# **Epidemiology of vector-borne diseases in cattle from SE Uganda**

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**PhD Thesis**

**The University of Edinburgh**

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## **Declaration**

I, Francis Awuor McOdimba, do declare that I composed this thesis, that it is my own work and that it has not been submitted for any other degree or professional qualification, except as specified.

Signed... ..



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....But He said to me, “  
”...II Cor. 12:9.

...And I answered “AMEN!!...Let it be so, Lord”

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## List of Abbreviations

AEZ	Agro-ecological zones
AFLP	Amplified fragment length polymorphism
°C	Degrees Celsius
CATT	Card agglutination test
CD	Clusters of differentiation
CCT	Capillary concentration technique
CFT	Complement fixation test
CI	Confidence interval
CK II	Casein kinase II
CL	Confidence limits
CNS	Central nervous system
CSF	Cerebro-spinal fluid
CTL	Cytotoxic T-lymphocytes
ddH <sub>2</sub> O	Double distilled water
DDT	4,4'-(2,2,2-trichloroethane-1,1-diyl)bis(chlorobenzane)
DFID	Department for International Development
DG	Dark ground
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
ECF	East Coast Fever
EDAC	1-ethyl-3 (3-dimethylaminesopropyl carbodiimine
EDTA	Ethylenedianinetetra acetic acid
EEC	European Economic Commission
ELISA	Enzyme linked immunosorbent assay
FAO	Food and Agriculture Organization
FITCA	Farming in Trypanosomiasis Controlled Areas
Hb	Haemoglobin
HCT	Haematocrit centrifugation technique
ICTTBD	Integrated control of ticks and tick-borne diseases
IFA	Indirect fluorescent antibody
IHA	Indirect haemagglutinin assay
IFAT	Indirect fluorescent antibody test
IL-2	Interleukin-2
ILRAD	International Laboratory for Research on Animal Diseases

ILRI	International Livestock Research Institute
ISMM	Isometamidium
ITM	Infection and treatment method
m-AECT	miniature-Anion Exchange centrifugation technique
(MGE)-PCR	(Mobile genetic element)- Polymerase chain reaction
MHC	Major histocompatibility complex
nm	nanometers
NF <sub>k</sub> B	Transcriptional activator nuclear factor B
PBS	Phosphate buffered saline
PCV	Packed cell volume
PCR	Polymerase chain reaction
PSG	Phosphate buffered saline with glucose
RAPD	Randomly amplified polymorphic deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
RLB	Reverse line blot
SIT	Sterile insect test
SRA	Serum resistance associated
SSA	sub-Saharan Africa
SS	Sleeping sickness
SSPE	Sodium phosphate buffered saline with EDTA
STDM	Standard trypanosome diagnostic methods
TBD	Tick-borne diseases
UDG	Uracil DNA glycosylase
UK	United Kingdom
US(A)	United States (of America)
US\$	United States dollar
UV	Ultra violet
VBD	Vector-borne diseases
VSG	Variable surface glycoprotein

## Abstract

Vector-borne diseases, (VBD), are amongst the most important constraints to animal production in tropical and sub-tropical regions of the world, including sub-Saharan Africa, (SSA). Losses due to these diseases are estimated to be up to 25% of the region's annual livestock production total. Some of the diseases are also zoonotic and therefore contribute to human health problems. The most important VBD are caused by the protozoa: *Anaplasma*, *Cowdria*, *Babesia*, *Theileria* and *Trypanosoma* parasites. *Anaplasma*, *Cowdria*, *Babesia* and *Theileria* are transmitted by ticks while trypanosomes are transmitted by tsetse flies in humans and animals in Africa. Control of these diseases in SSA is non-sustainable, mainly because of ineffective disease surveillance, poor diagnostic capacity and inadequate control measures largely due to economic constraints of the affected governments.

Two of the most commonly used diagnostic methods, microscopy and molecular techniques for pathogen detection and species characterization, were evaluated for their sensitivity and specificity. The results showed that the two techniques have very low diagnostic agreement in detecting all the three species of trypanosomes, (kappa values < 0.02). The sensitivity of PCR amplification in detecting trypanosomes in cattle blood was found to be 5-10 times higher than microscopy. The specificity of microscopy was found to be poor, in relation to PCR amplification as it mis-diagnosed many cases as having *T. vivax* while PCR showed that they were *T. brucei*. The study showed that *Theileria* species were more prevalent in Tororo than in Busia (60% vs. 38%). Molecular based methods revealed that *Theileria mutans* was the predominant species in cattle, at 42% prevalence while the overall prevalence of *Theileria* species was found to be 66% in Tororo district.

The prevalence of trypanosome species was found to be 10 times higher than previously recorded in this region. The study revealed that isometamidium chloride (Samorin) treatment of cattle did not protect the animals from infection with any of the three trypanosome species for more than three months. While Samorin treatment appeared to control trypanosomiasis in areas with low prevalence, the drug had no effect in controlling the disease in high prevalence areas. It would therefore be necessary to combine the use of drug intervention with other methods such as vector control, to reduce the prevalence, in order to realize effective control of trypanosomiasis. The results further show that Samorin treatment did not offer protection against *T. brucei*

infections in cattle. The study also showed that treatment of cattle with diminazene aceturate (Berenil) can not be relied upon to control transmission of trypanosomes. Finally, the study revealed that the prevalence of *T. brucei* is higher in adult animals than in calves while the prevalence of *T. congolense* and *T. vivax* is higher in animals below 24 months.

# CHAPTER ONE

## 1. LITERATURE REVIEW AND INTRODUCTION

### 1.1 IMPORTANCE OF LIVESTOCK IN SUB-SAHARAN AFRICA (SSA)

Sub-Saharan Africa's (SSA) human population is predicted to grow rapidly during the next 35 years to 1.3 billion. This poses a major challenge in terms of food sufficiency and calls for intensification of animal production, if Africa has to meet its increasing demand for livestock products from domestic resources. It has been estimated by the World Bank that around 10% of the population of SSA are primarily dependent on their animals, while another 58% depend to varying degrees on their livestock, (Winrock International, 1992).

Based on rainfall, altitude and length of the annual plant growing period, this region can be broadly divided into six agro-ecological zones: desert, arid, semi-arid, sub-humid, humid and highlands. Generally, the economic importance of livestock in African farming systems increases with decreasing rainfall, Table 1. For example, in Botswana with an arid type of climate, livestock constitute 82% of the value of agriculture, compared to 3.5% in the Democratic Republic of Congo and Uganda with humid and sub-humid type of climates respectively.

Table 1 Value of agriculture and livestock products in selected African countries including Uganda in1988, (US Department of Agriculture, 1990)

Country	Climate	Livestock value, (\$, millions)	Livestock share of agricultural output, , (\$, millions)
Botswana	Arid	107	88
Mauritania	Arid	158	84
Ethiopia	semi-arid	1,299	40
Kenya	semi-arid	826	38
Uganda	sub-humid	404	14
DR-Congo	Humid	143	5

However, the estimates of livestock values shown in Table 1 above do not include the value of manure and animal traction, which in East Africa, exceeds the value of meat (42% vs. 38%) and for SSA as a whole, would increase the total gross value of livestock products by

about 35% of agricultural domestic product (Winrock International, 1992). Livestock are an important part of African agriculture due to climate, terrain and traditional values of the farmer. They make a significant contribution to human nutrition in SSA, providing approximately 18% of total dietary protein as well as manure and urine that help in replenishing soil fertility for crop production. They are also used in animal traction thus increasing the productivity of smallholder farms.

Traditionally, agriculture in Africa is generally based on mixed crop-livestock systems, with pure livestock systems predominating when rainfall is too low and uncertain to support crop production. Similarly, pure arable systems only develop where diseases and parasites preclude livestock keeping. However, even in the so called pure livestock systems pastoralists usually practice some form of opportunistic seasonal cropping, while in primarily arable systems farmers tend to keep monogastrics or small ruminants as scavengers around the homestead. In mixed farming production systems livestock help improve the environment as farmers with livestock tend to plant forage trees, shrubs, grass contours and pasture, which prevent soil erosion and conserve water (Council for Agricultural Science and Technology, 1999) and are a major component in the intensification of agriculture and sustainability of crop production.

Livestock act as stores of protein and energy in the form of milk and meat that are consumed daily or in periods of food shortage, especially during crop failure. For many smallholder farmers livestock offer the only means of transferring from subsistence farming to a cash economy, generating a vital source of capital to purchase necessities, such as farm inputs (pesticides, seeds and fertilizers), pay for education of children and are used in exchange during marriages. Furthermore, crop sales can be highly seasonal, whereas livestock can generate a more regular income through sales of stock, milk and milk products (Kershaw, 1970; Jordan, 1986; Sansoucy, 1995).

## **1.2 DISEASE CONSTRAINTS TO LIVESTOCK PRODUCTION**

Animal diseases in tropical and subtropical regions of the world place a severe constraint on livestock productivity. In SSA, losses due to disease are estimated to be up to 25% of the region's annual livestock production total, (de Castro, 1997). Losses in productivity due

to disease are even more acute considering that food requirements in Africa, and other developing regions, could double or triple over the next 50 years due to population growth. Currently, the control of these diseases in SSA is non-sustainable due to ineffective disease surveillance, poor diagnostic capacity and inadequate control measures. Also, the local veterinary services and communal control facilities, such as cattle dips, are often poorly maintained (Winrock International, 1992), largely due to economic constraints of the affected governments. Livestock diseases not only cause losses due to mortality and morbidity of the affected animals, they also inflict significant costs for prevention and cure. Due to the multiple roles of livestock, diseases can also affect the growth of agricultural industry in general, resulting in lost development opportunities (Sansoucy, 1995).

In many countries in SSA improvement in milk and meat production are often sought by introduction of exotic taurine breeds of livestock and initiating cross- breeding. However, ‘improved’ livestock are usually more susceptible to the vector-borne diseases than the indigenous breeds found in SSA, Table 2, that frequently express adaptive traits for disease resistance.

Table.2 The main types of indigenous African cattle (Adapted from Leak, S.G.A. 1999)

Humpless cattle ( <i>Bos taurus</i> )		Humped cattle ( <i>Bos indicus</i> )	
Type	Examples	Type	Examples
Longhorns	Kuri, N'Dama,	West African zebu	Fulani
Shorthorns	West African Shorthorn	East African zebu	East African Shorthorn
		Sanga	Ankole
			Nilotic Sanga, Afrikander

### 1.3 VECTOR-BORNE DISEASES (VBD)

Vector-borne diseases are amongst the most important constraints to animal production in tropical and sub-tropical regions of the world, including SSA. Economically, the most important VBD are caused by the haemoparasitic protozoa: *Anaplasma*, *Cowdria*, *Babesia*, *Theileria* and *Trypanosoma* parasites. *Anaplasma*, *Cowdria*, *Babesia* and *Theileria* are



transmitted by ticks while trypanosomes are transmitted by tsetse flies in humans and animals in Africa, mechanically by triatomine bugs in humans in South America and transsexually in horses in many parts of the world including the Mediterranean, Asia and North Africa.

### **1.3.1 Diagnosis of vector-borne diseases**

Sensitive and specific diagnostic tests allow for the identification of pathogens that cause disease in animals and human patients. This in turn facilitates the prompt administration of appropriate intervention such as treatment or isolation of the affected individuals. Diagnostic tests are also important in identifying pathogens in order to understand transmission and control strategies.

Institutions involved in vector-borne diseases research, epidemiological studies as well as vaccine development require reliable and sensitive assays to support the development of vaccine products and new drugs for treatment. These diagnostic assays also aid in identifying disease control target populations, and to monitor infection during trials for the assessment efficacy of preventive or curative drug trials. Human hospitals and veterinary services as well as the private farming sector require diagnostics to identify susceptible individuals for immunization and to help plan strategies for the control of VBDs. Diagnostic tests are also important for identifying uninfected individuals in the surveillance surveys, to facilitate travel and trade within and between countries.

Over the years a number of diagnostic techniques have been developed to identify the organisms that cause VBDs. These techniques vary in sensitivity and specificity as well as in simplicity and cost and include parasitological, immunological and molecular diagnostic techniques.

#### **1.3.1.1 Parasitological diagnostic techniques**

In most parts of SSA, diagnosis of VBD is predominantly based on observation of characteristic clinical signs such as raised body temperatures, pale mucous membrane, ruffled coat and general body wasting and emaciation. Microscopic examination of body fluids such as blood and lymphatic biopsies are common for the direct identification of VBD causing agents. Although simple and inexpensive to perform, this method lacks

sensitivity and specificity and depends on the stage of the disease and the level of parasitosis. There is also a requirement for well trained and experienced personnel. The basic technique, for example in examination of fresh or stained blood films, for tsetse transmitted trypanosomes has been modified to improve diagnostic sensitivity by concentrating the blood through centrifugation in a haematocrit tube, namely the haematocrit centrifuge technique (HCT) or the dark ground buffy coat technique (DG) (Murray, *et al.*, 1979; Paris, *et al.*, 1982). While microscopy can detect parasites during patent infection, direct observation frequently fails to detect parasites in tick-borne disease carrier animals or cases of very low parasitaemia. Therefore microscopy is unsuitable for evaluating the role of carrier animals in the epidemiology of a disease. In particular, species and sub-species differentiation though fundamental in epidemiological studies, is in most cases impossible in the case of mixed infections by microscopy. This is exemplified by the difficulty in distinguishing *Trypanosoma brucei rhodesiense* pathogenic to humans from the non- pathogenic *T.b. brucei* as well as the difficulty in picking up very low infections in animals. Despite its many weaknesses, microscopy is still the most common technique in use for diagnosis of VBDs in many countries of SSA

#### **1.3.1.2 Immunological diagnostic techniques**

Although direct demonstration of trypanosomes in the infected animal gives conclusive proof of infection, the limitations of parasitological diagnostic techniques have led to the search for alternative techniques that provide indirect evidence of infection, namely immunodiagnostic techniques. Immunological methods such as indirect fluorescent antibody test (IFA) and enzyme-linked immunosorbent assay (ELISA) have been widely used for the detection of serum antibodies against specific pathogens in infected animals. There are many reports of the use of immunodiagnostic techniques for diagnosis but, invariably, most of them have been retrospective surveys, intended to add further information rather than play an integral part in a control programme. The one exception to this generalization is in the application of the complement fixation test (CFT) to the diagnosis of *T. equiperdum*, the cause of dourine in horses. CFT was used during the control and eradication of dourine in North America (Watson, 1920) and in the diagnosis of surra in buffalo in the Philippines (Randall and Schwartz, 1936). This assay is still in use for testing sera before the import and export of horses between different countries. However, the problems in the control and standardization of another sensitive test, the

indirect haemagglutination (IHA) test, have precluded the use of CFT, although it was used in the diagnosis of *T. evansi* in camels (Jatkar and Singh, 1971). In tests with *T. vivax* it was considered too unreliable (Clarkson, Cottrell and Enayat, 1971).

The breakthrough in immunological diagnosis came with the introduction of primary binding assays for the detection of pathogen antibodies. These tests directly measure the interaction between antigen and antibody rather than relying on a secondary reaction consequent upon the initial binding. The indirect fluorescent antibody test (IFAT) has been used extensively for the detection of *Theileria* as well as trypanosomal antibodies in animals and humans (Wilson, 1969; Luckins and Mehlitz, 1978) and camels (Luckins *et al.*, 1979).

However, studies have shown that cross-reactions between different trypanosome species do occur. Ashkar and Ochilo (1972) found that more than 85% of cattle infected with *T. vivax* or *T. congolense* reacted with *T. brucei* antigen in the IFAT. When sera were tested against all three pathogenic trypanosome species, between 45% and 66% of sera reacted in the assay, and only by combining all the results did the test detect 94% of infected animals; hence, although there is considerable cross-reactivity, these results indicate a degree of species specificity that requires the use of all three antigens in order to obtain maximum efficiency. Modifications in the preparation of antigens involving fixation of the parasites in acetone and formalin (Katende *et al.*, 1987) have provided antigens which are stable even at 4°C that can be kept in suspension until required and are capable of discriminating between different trypanosome species. The major drawback of the IFAT, other than its requiring sophisticated microscopy, is its subjectivity which can make comparison of results quite difficult.

The introduction of enzyme-linked immunosorbent assays (ELISA) for use as diagnostic tests for animal trypanosomiasis (Luckins and Mehlitz, 1978; Luckins *et al.*, 1979; Luckins, 1986; Rae, *et al.*, 1989; Ferenc, *et al.*, 1990) has increased interest in the possibility of a universally applied immunodiagnostic assay, and the modification and refinement of these assays brings field tests a little closer (Nantulya *et al.*, 1989).

The tests described above rely on antigen/antibody reactions between common or species-specific antigens. However, the card agglutination test (CATT) uses the formalin fixed variable antigen types of *T. evansi* that are used in the agglutination test. The test, which is simple to perform, has been used for diagnosis of *T. evansi* (Bajyana-Songa *et al.*, 1987). A modification of the ELISA (antigen ELISA), based on an antigen capture assay which enables detection of circulating parasite antigen in the blood of infected animals was developed and shown to have a high diagnostic sensitivity; more than 90% in cattle and 95% in camels infected with trypanosomes (Nantulya *et al.*, 1989; Nantulya, 1990). However, false negative results occurred during the early stages of infection, possibly when antigen levels were below the detection limits of the assay. Although simple and fairly specific, these methods do not distinguish between past and current infections even after drug treatment due to the circulating antigen molecules in the animal which take some time to clear and are therefore unsuitable for epidemiological evaluation of VBDs.

#### **1.3.1.3 Molecular diagnostic techniques**

Organisms have variations at the DNA sequence level that underlie the observed phenotypic differences between species. However, some neutral DNA variations that are not phenotypically observable exist in multiple repeats in the genome and are useful for molecular characterization of different species as well as differences in intra-species. Molecular diagnostic techniques demonstrate some of these unique DNA molecules that are specific to individual pathogen species, strains or types. These techniques have proved valuable in improving sensitivity and in distinguishing species and sub-species as well as strain typing of various pathogens which would otherwise be difficult or impossible to distinguish microscopically or serologically. Broadly speaking, there are three formats of DNA-based molecular diagnostic techniques:

- Techniques based on hybridization of cloned DNA probes or known synthetic oligonucleotides to parasite nucleic acids, (Conrad, *et al.*, 1987a).
- Techniques based on the detection of parasite DNA using the polymerase chain reaction (PCR) amplification with specific primers, (Masiga, *et al.*, 1992).
- A combination of PCR amplification and hybridization, such as the reverse line blot, (Gubbels, *et al.*, 1999).

The major drawback of DNA probes using hybridization methods such as Southern hybridization analysis and restriction fragment length polymorphism, (RFLP) is that they require relatively large quantities of purified DNA or ribosomal RNA. The application of polymerase chain reaction (PCR) amplification (Saiki, *et al.*, 1988) using specific oligonucleotide primers derived from repetitive sequences of both tick and tsetse transmitted parasites has been successful in detecting parasites of several species, sub-species and types. PCR-based assays for detecting parasites in carrier state cattle or those under persistent infection with *Theileria* and trypanosomes are currently favoured over microscopic examination of blood or immunological methods because of their superior sensitivity and higher throughput (Gubbels, *et al.*, 1999).

## **1.4 TSETSE-BORNE DISEASES**

Tsetse-borne diseases, collectively known as trypanosomiasis, are a group of diseases of great medical and economic importance throughout Africa, South America and Asia. In man, the disease (also known as sleeping sickness in Africa and Chagas' disease in South America) causes devastating ill health and social disturbance. In domestic livestock the disease, known in various places as nagana, Surra, mal de caderas, murina, derrengadera or dourine, is responsible for widespread malnutrition due to losses in milk and beef production, (Kershaw, 1970), as well as diminished traction power of the animals in farming. The impact of tsetse infestation and trypanosomiasis on health and economic loss is greater in SSA where it has led people to avoid livestock farming in more fertile regions, limiting themselves to the more arid areas (Jordan, 1986).

### **1.4.1 Classification of trypanosomes**

Trypanosomes, (Figure 1), are unicellular flagellate protozoa of the genus *Trypanosoma*. The important members of this genus that infect man and animals are listed in Figure 2 (Vickerman, 1963; Levine *et al.*, 1980).



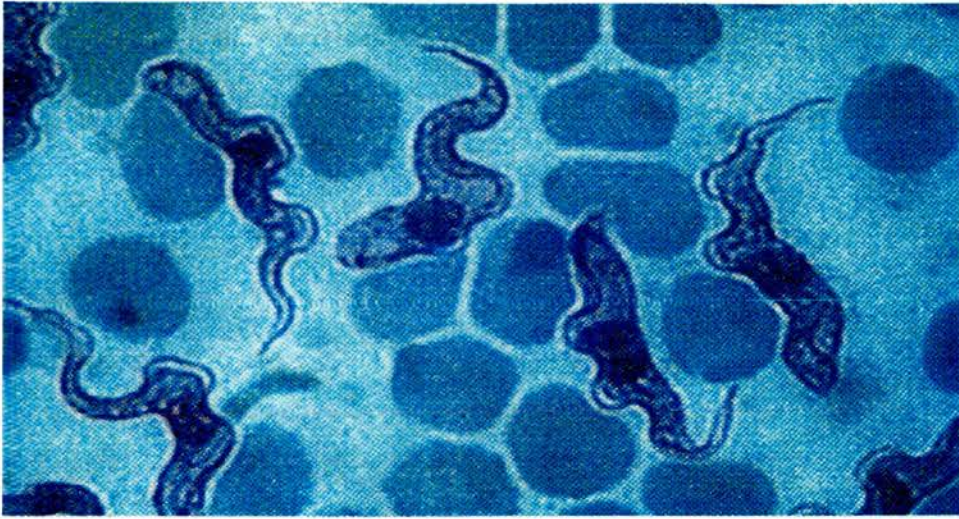


Figure1: Bloodstream forms of *Trypanosoma brucei brucei*. (Picture Max Murray)

The mammalian parasites are classified as Stercoraria and Salivaria groups, (Figure 2).

- The Stercoraria develop in the hindgut of the triatomine vector and their transmission is by faecal contamination of wounds.
- In the Salivaria groups, the infective stages of the parasites are found on the mouthparts of tsetse and tabanid flies and their transmission is through fly bites.

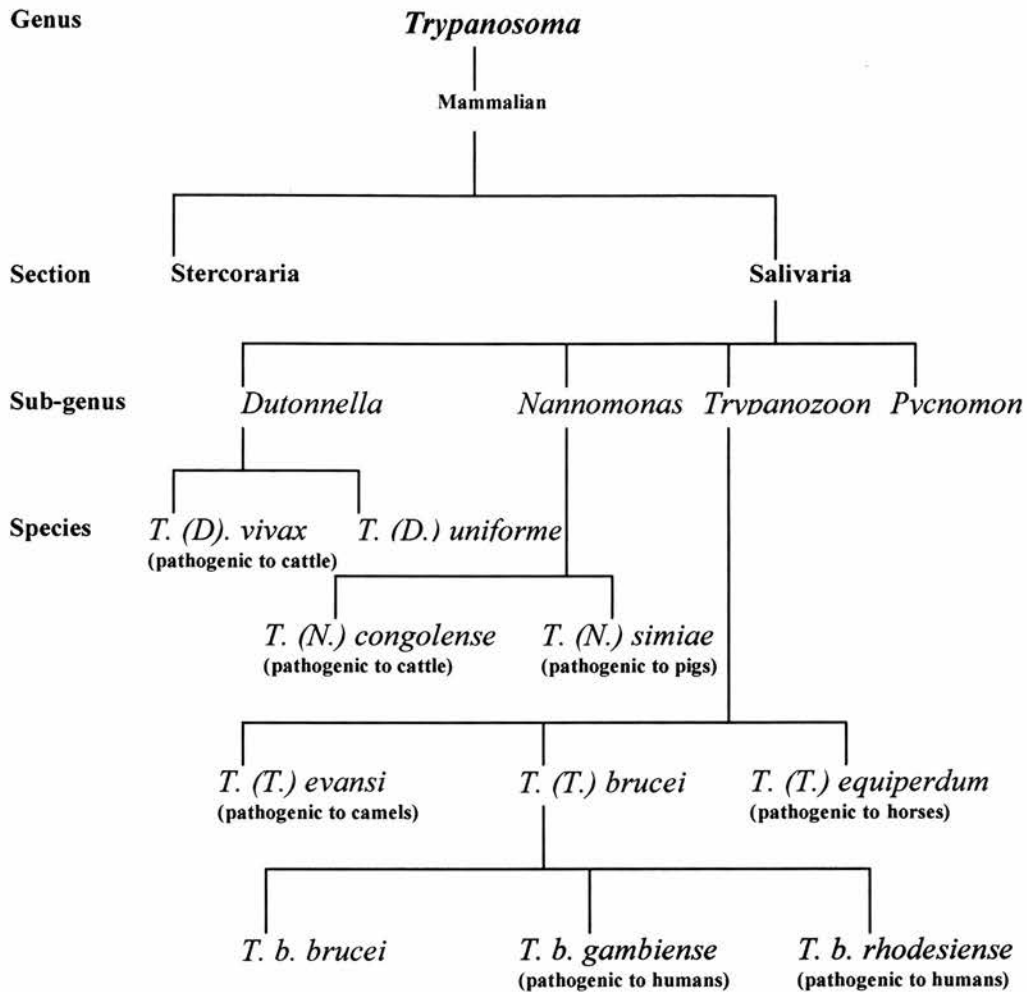


Figure 2: Classification of mammalian infective trypanosomes

#### 1.4.2 Structure of Trypanosomes

In spite of slight differences between the various trypanosome species, they are predominantly spindle-shaped and flat-bodied organisms ranging in size from  $10\mu$ , (*T. congolense*) to  $120\mu$  (*T. megatrypanum*), depending on the species. The lengths of trypanosomes range from 8 to  $42\mu$ m, as measured from anterior to posterior. These parasites possess certain distinct morphological features and organelles (Mulligan, 1970) (Figure 3) the location and presence or absence of which can be used to classify the various species and differentiate the various life cycle stages.

They use their flagella as a means of locomotion and some species such as *T. brucei* have a long free flagellum while in others, such as *T. congolense*, it is short. From its posterior origin the flagellum extends anteriorly and is attached to the cell body by a thin pellicular film known as the undulating membrane, which is prominent in some species such as *T. brucei* and almost absent in others such as *T. congolense*.

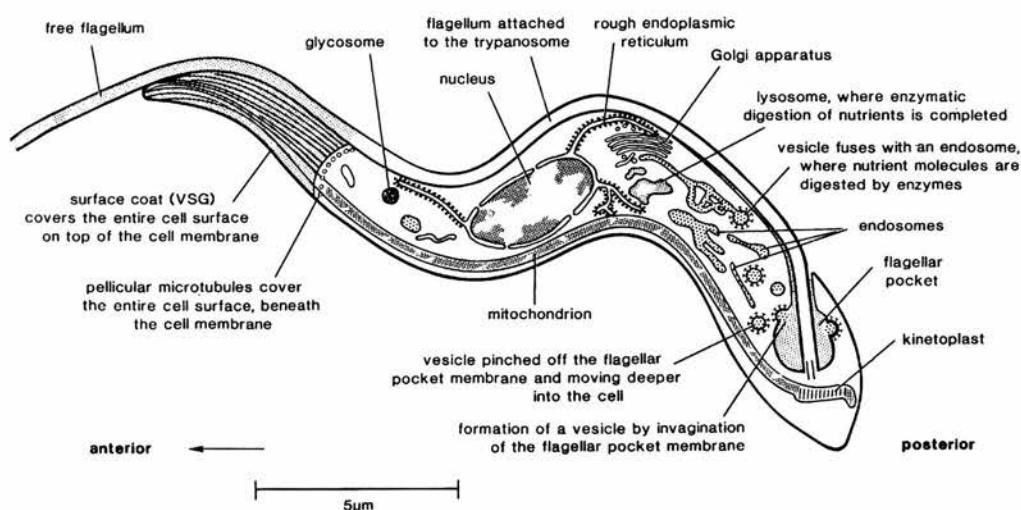


Figure 3: Schematic diagram of a bloodstream form trypanosome illustrating the major organelles. Adapted from Vickerman, (1970)

At the anterior end the flagellum may terminate, as is the case with *T. congolense*, or extend freely as is the case with *T. brucei*. In addition to its role in motility, the flagellum is also involved in parasite adhesion to red blood cells surfaces as is clearly seen in *T. congolense*, (Banks, 1979 ), as well as in trafficking of nutrients and secretory products through the flagellar pocket (Vickerman and Preston, 1976; Vickerman and Tetley, 1979). The other major structural organelles of the trypanosome include a nucleus and a posteriorly located kinetoplast in close proximity to a pair of basal bodies. Both pinocytosis and receptor-mediated endocytosis are believed to occur within the flagellar pocket, with the latter process utilizing specialized ‘coated pits’.



### 1.4.3 The life cycle and development of trypanosomes

Salivarian trypanosomes are zoonotic in Africa, where *Trypanosoma brucei gambiense* and *T. b. rhodesiense* are responsible for human trypanosomiasis or sleeping sickness while *T. b. brucei*, *T. vivax* and *T. congolense* cause animal trypanosomiasis. The infection of humans or cattle and the subsequent occurrence of trypanosomiasis form a significant part of the cyclical development of the pathogenic Salivarian trypanosomes (Figure 4). The trypanosome stage found within humans or animals are bloodstream form trypomastigotes a rapidly proliferating form.

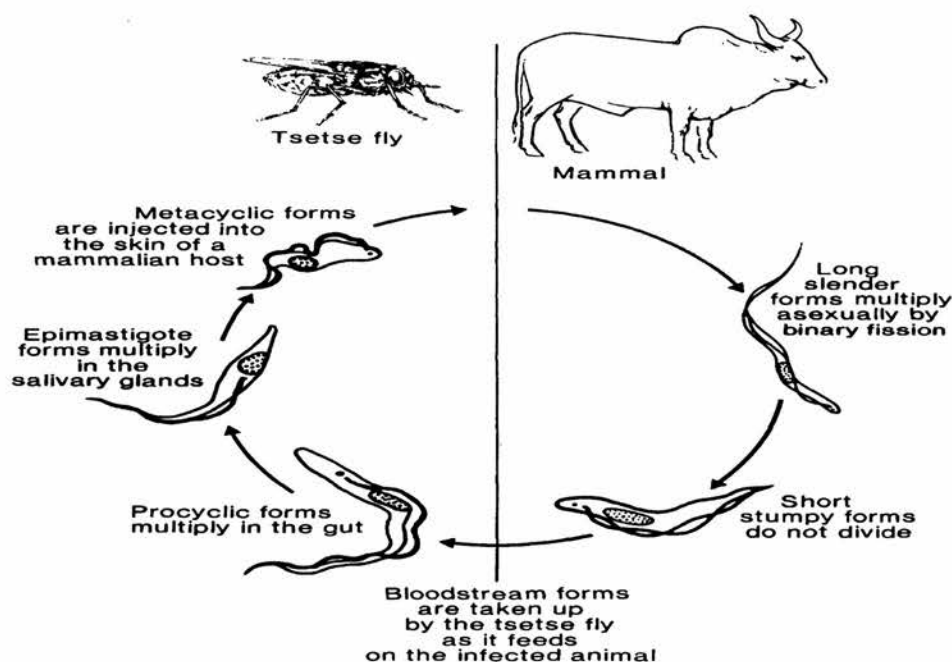


Figure 4: Cyclical transmission of Trypanosomes in the vector and mammalian host

From the mammalian host trypanosomes continue their developmental process within the tsetse fly (*Glossina spp.*) after the insect takes up blood from the infected host. Once the tsetse fly bites an infected animal host it will remain infected for the rest of its life. Following multiplication, migration and maturation of the trypanosomes, the fly then becomes infective to the next mammalian host on which it feeds.

The location and duration for completion of trypanosome cyclical development within the tsetse fly (Figure 5) varies between the different trypanosome species. This cycle ranges from 5 days in flies infected with *T. vivax* to 2 – 3 weeks and 3 – 5 weeks for infections with *T. congolense* and *T. brucei* respectively (Vickerman *et al.* 1988).

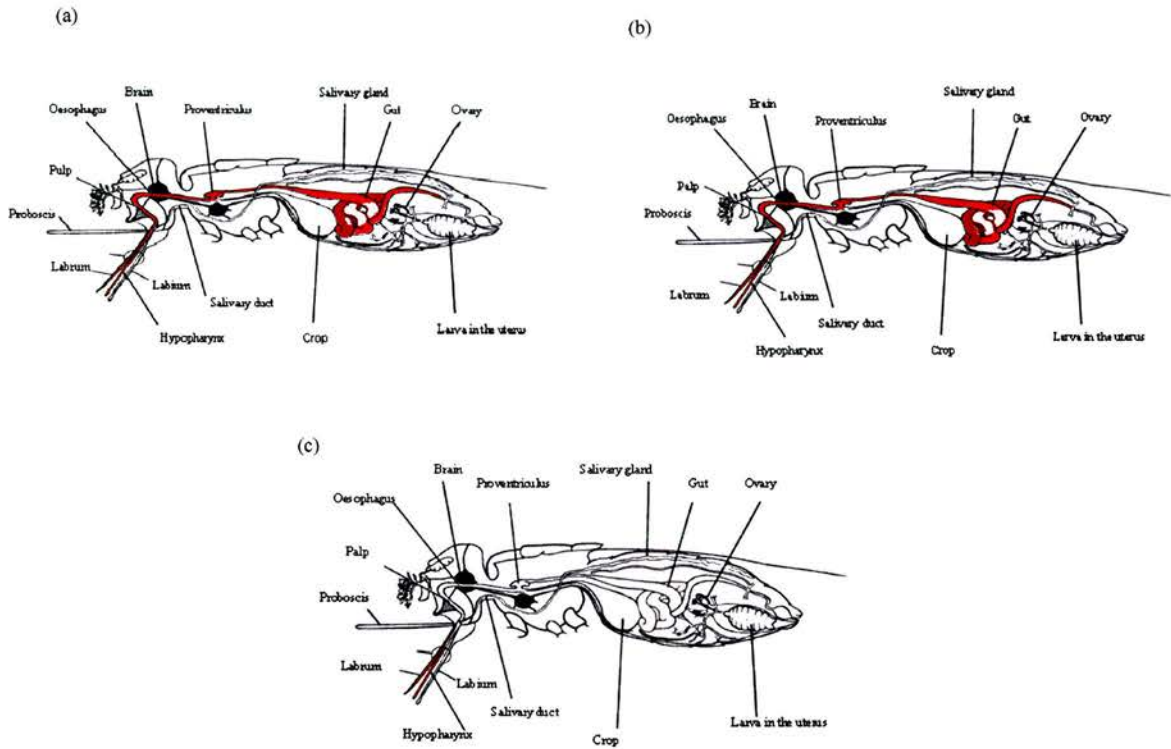


Figure 5: Development of various trypanosome species in the *Glossina* vector, (a)- *T. brucei*; (b) - *T. congolense*; (c) - *T. vivax*. Courtesy of ILRI Graphics Department

#### 1.4.4 Trypanosome species of medical and veterinary importance

##### 1.4.4.1 *Trypanosoma (Trypanozoon) brucei* (Plimmer and Bradford 1899)

These are pleomorphic trypanosomes that show distinct slender, intermediate and stumpy forms. The slender forms are thin and long, (average 35  $\mu\text{m}$ ) with a long free flagellum. The stumpy forms are about 18 $\mu\text{m}$  and are broad but lack the free flagellum. The intermediate forms are in-between and always have a free flagellum. All forms have a clear undulating membrane but their kinetoplasts are small.

In their development, the trypomastigotes within the proboscis move through the salivary duct and into the salivary gland of the fly where they attach to the epithelial wall as epimastigotes before maturing to coated metacyclic trypomastigotes (Figure 5a). These are then released into the animal along with the saliva by the fly during feeding (Vickerman *et al.*, 1988). The trypanosomes in this group are transmitted by tsetse flies. It is only in preparation for transmission into the insect vector that multiplication by binary fission ceases. The short, stumpy trypomastigotes are predominantly the infective stage for *T. brucei* parasites to the insect vector.

*Trypanosoma brucei* species contains sub-species, *T.b. rhodesiense* and *T.b. gambiense*, (Figure 2) that cause human sleeping sickness. The two subspecies along with *T. brucei* are indistinguishable from each other by microscopy. However, by using human serum lysis test, the morphologically identical *T.b. brucei* can be differentiated from *T.b. rhodesiense*, by incubation of the parasites with human serum, since the former can be lysed by human serum while the later is resistant, (Hawking, 1967).

### **The SRA gene**

The *Trypanosoma brucei* s.l. genome contains variable surface glycoprotein (VSG) genes that have some sequence identity with the serum resistance associated (SRA) gene. When the SRA gene is compared with the VSG genes it does appear that the SRA gene has undergone some sequence deletion of about 378 base pairs that encode the central part of the VSG N-terminal domain (Figure 6a) (Campillo and Carrington, 2003).

Recently, a gene that codes for the serum resistance associated glycoprotein (Figure 6) has been cloned from *T.b. rhodesiense* (De Greef, *et al.*, 1989) and has been used as a molecular marker to distinguish this sub-species from the other *Trypanozoon*. This gene known as SRA gene has been used in PCR assays to screen for the human infective parasites in cattle (Welburn, *et al.*, 2001). All the sub-species of *T. brucei* s.l. exist in Uganda and both *T. b. brucei* and *T. b. rhodesiense* sub-species exist in SE Uganda where it has long been established that transmission to cattle can occur with no apparent serious pathological results. Indeed it has been documented that cattle can act as reservoir hosts for *T. b. rhodesiense* that causes the acute form of human sleeping sickness, (Onyango, *et al.*, 1969).

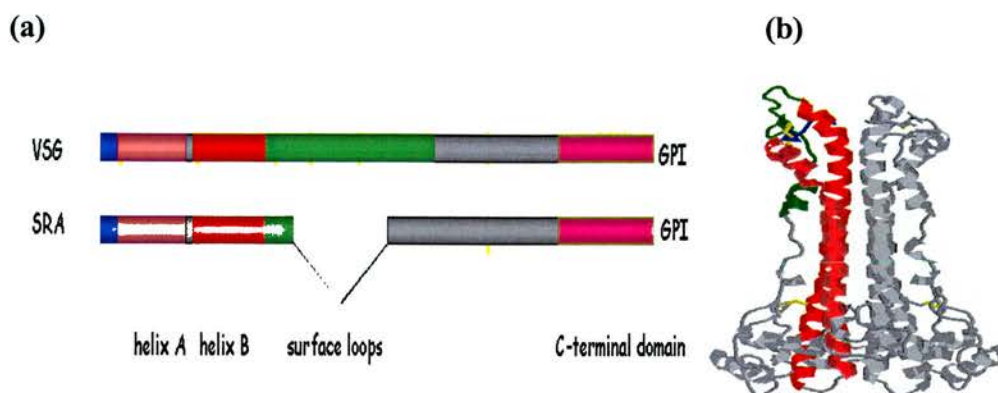


Figure 6 (a) Structure of VSG compared to SRA gene and (b) SRA protein structure. Adapted from Carrington, 2003.

#### 1.4.4.2 *Trypanosoma nannomonas congolense* (Broden 1904)

This is a small parasite of about 9-18 $\mu$ m in length and lacks a free flagellum but has a medium size kinetoplast. In nannomonas group, which contains *T. congolense*, trypomastigotes develop into epimastigotes that attach to the walls of the labrum and hypopharynx of the tsetse vector (Figure 5b) where they mature into metacyclic forms that are ready to be injected into the animal host. *T. congolense* is one of the most important species that cause animal trypanosomiasis in Africa, being responsible for the chronic form of the disease in cattle; it is transmitted exclusively by tsetse flies. The species comprises parasites that are morphologically identical when viewed under the microscope but with varied pathogenicities. However, based on their pathogenicity and genetic variability, several different types of this parasite have been identified in tsetse flies as well as in wildlife (Godfrey, 1981; Young and Godfrey, 1983; Majiwa, *et al.*, 1985; Majiwa, *et al.*, 1986; Knowles, *et al.*, 1988;). These include *T. congolense* Savannah-type; the West African forest/riverine type; the Kilifi-type and more recently the Tsavo-type (Young and Godfrey, 1983; Majiwa, *et al.*, 1985; Majiwa, *et al.*, 1986; Knowles, *et al.*, 1988; Majiwa *et al.*, 1993).

The Tsavo-type *T. congolense*, isolated for the first time from *Glossina pallidipase* in Tsavo West National Park in Kenya, (Mihok, *et al.*, 1992), was initially described by Majiwa and others (1993), after comparative evaluation of various species and sub-species



of trypanosomes by using repetitive DNA sequences specific for the various types of African trypanosomes (Majiwa, *et al.*, 1985; Gibson, *et al.*, 1988). Subsequent phylogenetic studies showed that this type of *T. congolense* was closer to *T. godfreyi* and *T. simiae* than to the other types of *T. congolense* (Stevens, *et al.*, 1998). *Trypanosoma godfreyi* and *T. simiae* are however not known to be pathogenic to cattle (Garside, and Gibson, 1995). While the Tsavo-type of *T. congolense* has been isolated from tsetse flies and wildlife, there is no literature indicating its presence in cattle and therefore its pathogenicity is not known.

#### **1.4.4.3 *Trypanosoma duttonella vivax* (Ziemann 1905)**

This is a relatively large trypanosome that is about 20 – 26 µm in length with a free flagellum and a large terminal kinetoplast situated close to the posterior end. It is the main cause of acute trypanosomiasis in cattle. The life cycle of *T. vivax* is restricted to the labrum and hypopharynx in tsetse fly. The majority of the aspirated *T. vivax* non-dividing bloodstream form trypomastigotes are swept through the pharynx, oesophagus and proventriculus into the crop. These are later released into the mid-gut where it has been shown that they die within 2 to 3 days (Moloo and Gray, 1989). A few trypomastigotes get attached to the labrum by their flagella (Gardiner, *et al.*, 1986). It is here within the proboscis of the fly (Figure 5c) that bloodstream trypomastigotes differentiate to epimastigotes resulting in the characteristic *T. vivax* rosette clusters (Vickerman, 1973). Epimastigotes differ from trypomastigote forms with regard to the anterior localization of their kinetoplast and flagellar pocket in addition to possessing a more emergent flagellum. Once they mature, the parasites migrate to the hypopharynx where they differentiate to metacyclic trypomastigotes, acquire the variant surface glycoprotein (VSG) coat and are ready for the mammalian host. *T. vivax* is transmitted by tsetse flies but can also be transmitted mechanically through bites of other insects such as tabanids (Desquesnes and Dia, 2004).

#### 1.4.5 Epidemiology of African animal trypanosomiasis

The distribution of trypanosome-infected and potentially infected livestock in SSA is closely related to that of tsetse species of the genus *Glossina*, which are the prime vectors of the disease. The ecological limit of *Glossina* distribution is from 14 °North from Senegal in the West to 10°North in southern Somalia in the east and latitude 20°S, to the northern fringes of the Kalahari and Namibian deserts (Figure 7) (Rogers and Randolph, 1991). This is an area of about 11 million km<sup>2</sup> spread over 37 countries, or half of the available arable land of SSA. The limits of tsetse infestation are primarily determined by climate since temperature and vegetation are very important parameters as the distribution of the tsetse fly vector shows.

There is a large body of experimental evidence to show that host preferences and vector capacity differ greatly between groups and species of *Glossina*. For example, recent studies with teneral tsetse flies in Burkina Faso have shown higher mature infection rates with the savannah type of *T. congolense* in *G. morsitans morsitans*, (Ulienbergh, 1998) and *G. morsitans submorsitans* (both belonging to the savannah group of tsetse) than in *G. palpalis gambiensis* and *G. tachinoides* (*palpalis* or riverine group). *G. morsitans submorsitans* was the best vector of both the savannah and the riverine-forest types of *T. congolense*, while *G. m. morsitans* had the lowest vectorial capacity for the riverine-forest type; *G. palpalis gambiensis* was the least effective vector for the savannah type of *T. congolense*. In general, Savannah species are better vectors of the pathogenic trypanosomes of livestock. Also, where savannah tsetse (the *morsitans*) group are the vectors, the risk of contracting the disease is widespread, although their distribution area in the dry season decreases.

When riverine species are dominant, such as in many parts of West and Central Africa, transmission occurs particularly along rivers with dense vegetation along the banks (gallery forests). Some of the forest species such as the *fusca* group are confined to dense forest and are therefore not normally in contact with livestock, but some also occur on the forest edge and may locally play a significant role as vectors of African animal trypanosomiasis. Populations of savannah species feed mainly on mammalian hosts, particularly bovids such as the antelopes, buffalo, cattle, sheep, goats and suids such as the warthogs and bush pigs,

while the riverine tsetse have a very wide range of preferred hosts, including reptiles and humans. Zebras, certain antelopes and also carnivores have little attraction for tsetse flies. The proportion of a tsetse population found infected with pathogenic trypanosomes therefore depends not only on its vector capability, but also on the hosts on which it mainly feeds. For instance, reptiles do not carry pathogenic trypanosomes (Ulienbergh, 1998) and there are also major differences between suids and bovids, as the former will infect the flies particularly with *T. simiae* and *T. godfreyi*, while bovids are mainly the source of *T. vivax* and *T. congolense*, (Ulienbergh, 1998).

Herd management is also important since the daily activity patterns of the tsetse species involved and the grazing patterns of the herds are of great influence. If the herds graze on infested sites at the time of the day that flies are most active, transmission will occur more frequently. In the Sahel zone, many of the cattle owners, such as the Baggara and the Fulani, are transhumant because in the dry season the pastures and watering places in the Sahel are insufficient to maintain the large livestock populations. The zebu herds, accompanied by small ruminants, are then moved hundreds of kilometres to the south, where they may enter tsetse belts and contract the disease. Although the owners generally know the danger and recognize and associate tsetse flies with the disease, they are not always able to avoid infested areas. Particularly during dry years the southward migration is greater than usual, and the owners may deliberately choose between the risk of starvation of the herd and of tsetse-transmitted Trypanosomiasis (Ulienbergh, 1998). At the beginning of the rainy season transhumants start to move back to the Sahel pastures, in order to arrive when these are sufficiently lush. The animals infected in the tsetse belts are diseased by the time they reach the rainy season pastures and may even die, the physical effort of transhumance adversely affecting the outcome. Unless the animals are treated in time, great losses may occur and when there are large numbers of tabanids and other biting flies around during the rains, the infection may be further transmitted mechanically outside the tsetse belts.

Species and breed susceptibility are important and whereas in tsetse areas Trypanosomiasis is a very obvious problem in susceptible livestock, it may remain inapparent where trypanotolerant breeds are concerned. The risk to susceptible ruminants living in comparatively tsetse-free areas surrounded by tsetse-infested regions, or at the edge of tsetse-belts, varies from year to year. Generally, tsetse populations during wet years will increase, spread, and persist during the dry season in areas from where they disappear in dry years. Also, animals used for transporting persons or goods are particularly at risk. For example, although the classical breeding areas of camels in Africa are north of the tsetse belts, individual camels are used for the transport of merchandise to transhumant animal owners in their dry season grazing grounds in or near tsetse belts, and these camels risk contracting tsetse-transmitted Trypanosomiasis. Camels are highly susceptible to *T. congolense* and to *T. brucei* infections but do not usually penetrate into tsetse areas. The same applies to the riding horses of travellers and of transhumant cattle owners.

The epidemiology of non tsetse-transmitted trypanosomiasis such as *T. evansi* and sometimes *T. vivax* is also influenced by many factors. There may be seasonal outbreaks, where the populations of biting flies (Tabanids, stable flies, etc.) are influenced by important seasonal climatic differences. The disease sometimes becomes more clinically apparent during the dry season, when immuno-depressive factors such as poor nutritional state of the animal diminishes its defences, even when the initial infection occurred during the rains. The epidemiology is also influenced by host preferences and diurnal behaviour patterns of the various local species of tabanids and other biting flies. The main reservoirs of *T. vivax* infection in Latin America are probably domestic ruminants themselves, but *T. evansi* has found new wild reservoirs such as blood-sucking vampire bats and the capybara, a giant rodent, (Ulienbergh, 1998).



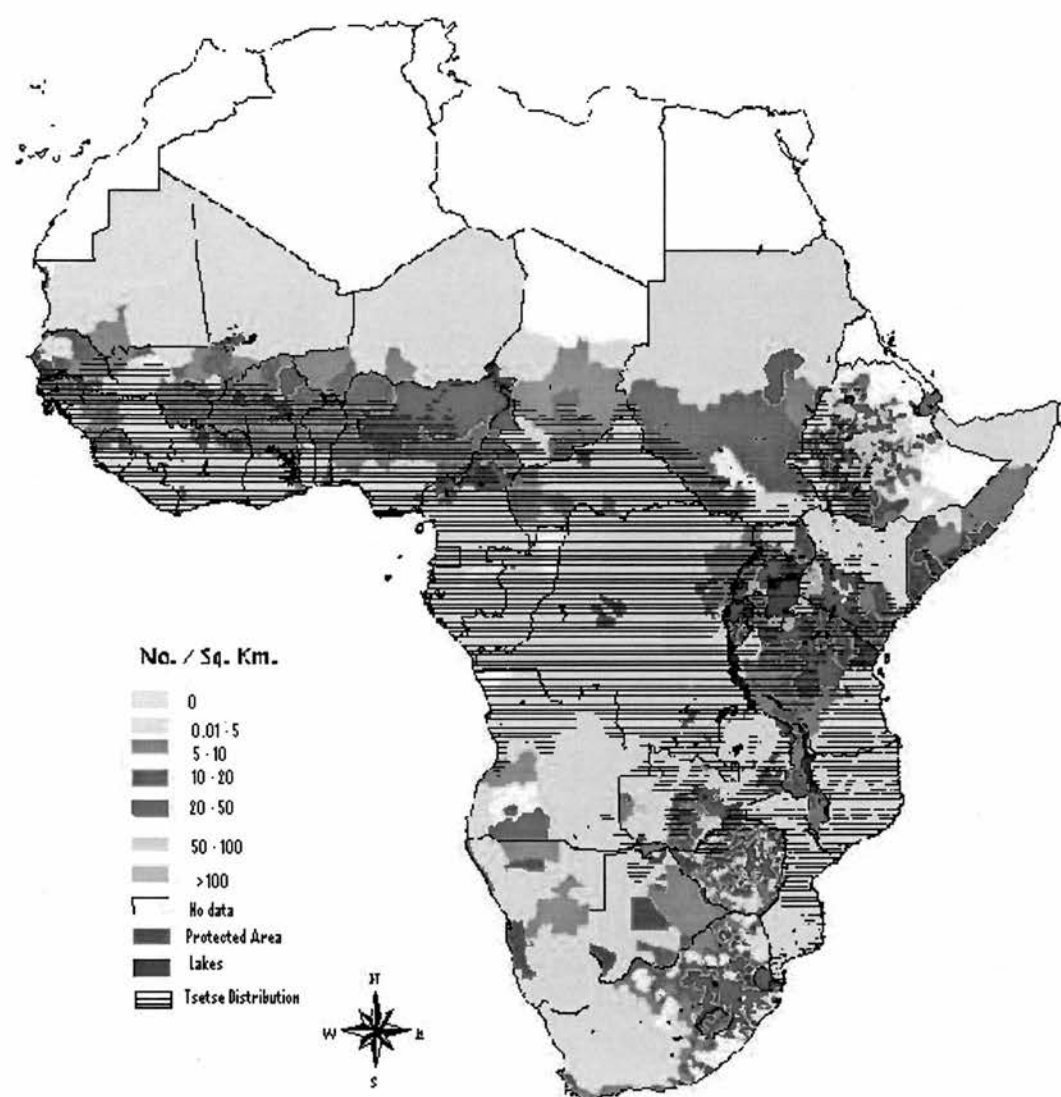


Figure 7: Map showing cattle rearing areas and tsetse distribution in Africa. The overlay of tsetse distribution with cattle density (coloured areas) is shown on the map. The abbreviation No/sq.km, is number of cattle per square kilometre depicting the cattle density in various parts of Africa. (Map drawn by Russ Kruska, ILRI)

Table 3: The occurrence of African trypanosomes in domestic and wild animals

Trypanosome species	Domestic animals affected	Reservoir hosts	Laboratory animals
<i>T. congolense</i>	Cattle, camels, horses, dogs, sheep, goats, pigs	Several groups of wild mammals	Rats, mice, guinea pigs, rabbits
<i>T. simiae</i>	Pigs	Wart hog, bush pig	Rabbits, monkeys
<i>T. godfreyi</i>	Pigs	Wart hog	None susceptible
<i>T. vivax</i>	Cattle, sheep, goats, domestic buffalo, horses	Several groups of wild mammals	Usually none susceptible
<i>T. uniforme</i>	Cattle, sheep, goats	Various wild ruminants	None susceptible
<i>T. brucei brucei</i>	Horses, camels, dogs, sheep, goats, cattle, pigs	Several groups of wild mammals	Rats, mice, guinea pigs, rabbits
<i>T. brucei gambiense</i> , <i>T. brucei rhodesiense</i>	Human sleeping sickness; affect domestic animals as <i>T. brucei brucei</i>	Several groups of wild mammals (particularly <i>T. brucei rhodesiense</i> )	As for <i>T. brucei brucei</i> (after initial adaptation where <i>T. brucei gambiense</i> is concerned)
<i>T. evansi</i>	Camels, horses, dogs, domestic buffalo, cattle	Several wild mammals in Latin America	As for <i>T. brucei brucei</i>
<i>T. equiperdum</i>	Horses, donkeys, mules	Not known	As for <i>T. brucei brucei</i> (after initial adaptation)
<i>T. theileri</i> and <i>T. ingens</i> (subgenus <i>Megatrypanum</i> )	Cattle, domestic buffalo (not pathogenic)	Various wild ruminants	None

#### 1.4.6 Economic importance of trypanosomiasis

Of all diseases affecting animals in Africa, trypanosomiasis is considered to be the most important (Jawara, 1990). It has been estimated that the annual losses due to trypanosomiasis in Africa is about U.S \$ 4 - 5 billion, (Budd, 1999; cited in Eisler, *et al.*, 2003). Trypanosomiasis seriously constrains development by direct economic and social losses in terms of both livestock and human mortalities and morbidity arising as a result of exposure to disease. Nagana, the form of trypanosomiasis affecting cattle in Africa, is of more importance economically than sleeping sickness, which affects humans, and hence emphasis is mainly on this disease.

Indirectly, reduced productivity and social losses are evidenced in rural communities but ultimately this affects the national economy (Chadenga, 1994). Losses in milk production, beef production, draught power and useful by-products such as hides and skins are enormous. Furthermore, due to impaired fertility in trypanosome infected animals (Sekoni *et al*, 2004), fewer animals are available for slaughter and sale. As a consequence of the low

birth rate, fewer animals lactate thus reducing milk production. These indirect losses due to trypanosomiasis are difficult to quantify, but are only a part of the true cost of the disease. The cumulative effect of lower milk production, reduced work output, lower crop yields and impaired local transportation impede the development of affected areas (Connor, 1994). The costs of controlling the disease also contribute to the economic losses incurred since the different methods in use are expensive and finances that could be used elsewhere are instead spent on controlling the disease.

#### **1.4.7 Trypanosome hosts**

Trypanosomes have a diverse range of hosts, both vertebrates and invertebrates. Among the invertebrates which also serve as vectors are the *Glossina*, and the tabanid bugs. The parasites are however quite ubiquitous in terms of vertebrate hosts, some of which serve as reservoir hosts. They infect just about any animal that the infected vector feeds on including reptiles and mammals, both domesticated and wild animals (Ford, 1970). In addition to the pathogenic species transmitted by tsetse flies, in Africa animals may also harbour non-pathogenic trypanosomes such as *T. theileri* and *T. ingens* which are found in both domestic and wild ruminants (Table 3); *T. lewisi* and *T. musculi* in rodents; *T. vespertilionis* and *T. megadermae* in bats, (Hoare, 1970).

##### **1.4.7.1 Trypanosome vector hosts**

Tsetse flies of the genus, *Glossina* (Figure 8) which are the main vectors for African trypanosomiasis, belong to the family Glossinidae, order *Diptera* also known as the two-winged flies. There are 31 species and subspecies in this genus so far identified which can be broadly categorized into three main groups according to their genitalia: the morsitans group which predominantly inhabit the Savannah type of vegetation, the palpalis group, which inhabit the gallery forest type of vegetation and the fusca group which inhabit the forest type of vegetation (Figure 9) (Ford, 1970, 1971; Jordan 1988).

The *fusca* group are primarily vectors for wildlife infections and are found mainly in humid forest zones where the domestic livestock population is low. This group includes the flies whose female genitalia are described as having inferior signum (Mulligan, 1970). Members of this group include *G. fusca fusca*, *G. nigrofusca nigrofusca*, *G. nigrofusca hopkinsi*, *G. medicorum*, *G. tabaniformis*, *G. brevipalpis*, *G. longipennis*, *G. frezili*, *G. severini*, *G.*

*haningtoni*, *G. fuscipleuris* and *G. vanhoofi*. In countries such as Gabon and Democratic Republic of Congo (DRC), where N'Dama cattle are reared within forest areas, tsetse flies from this group are important in the transmission of trypanosomiasis (Leak *et al* 1991). However, the encroachment of forests by man for timber and cultivation continues to reduce the formerly wide distribution of the *fusca* group to isolated pockets.

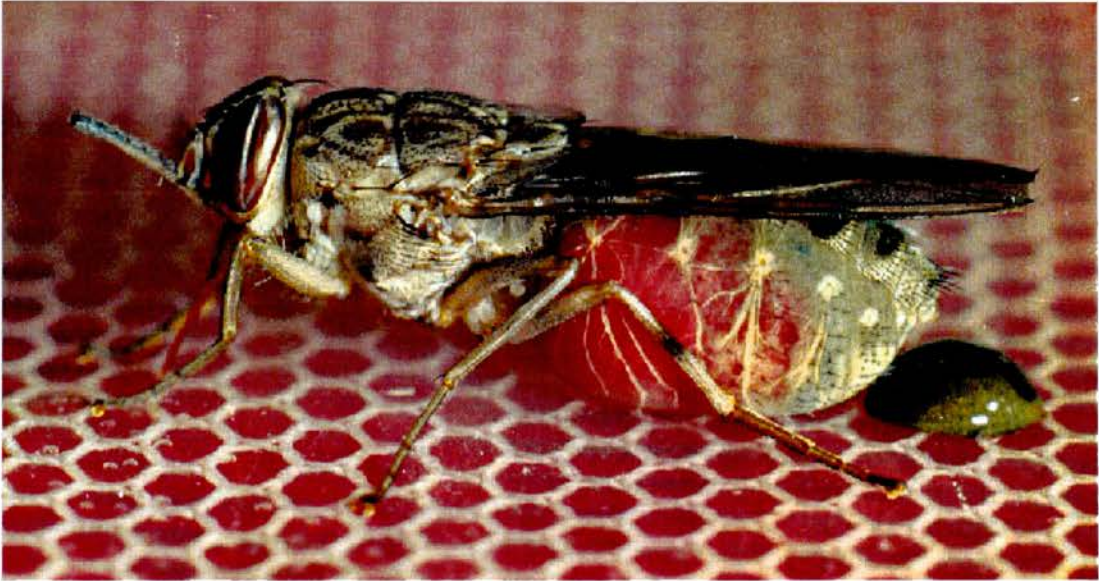


Figure 8: Tsetse fly feeding on blood through a nylon membrane. (Courtesy, ILRI Graphics)

The *G. morsitans* group mainly inhabits the savannah woodlands which are the prime areas for grazing livestock and are the most efficient and common vectors for both animal trypanosomiasis and human sleeping sickness. This group includes the flies whose genitalia are characterized as having superior claspers (Mulligan, 1970). Members of this group include *G. morsitans submorsitans*, *G. morsitans centralis*, *G. morsitans morsitans*, *G. austeni*, *G. pallidipes*, *G. swinertoni* and *G. longipalpis*.

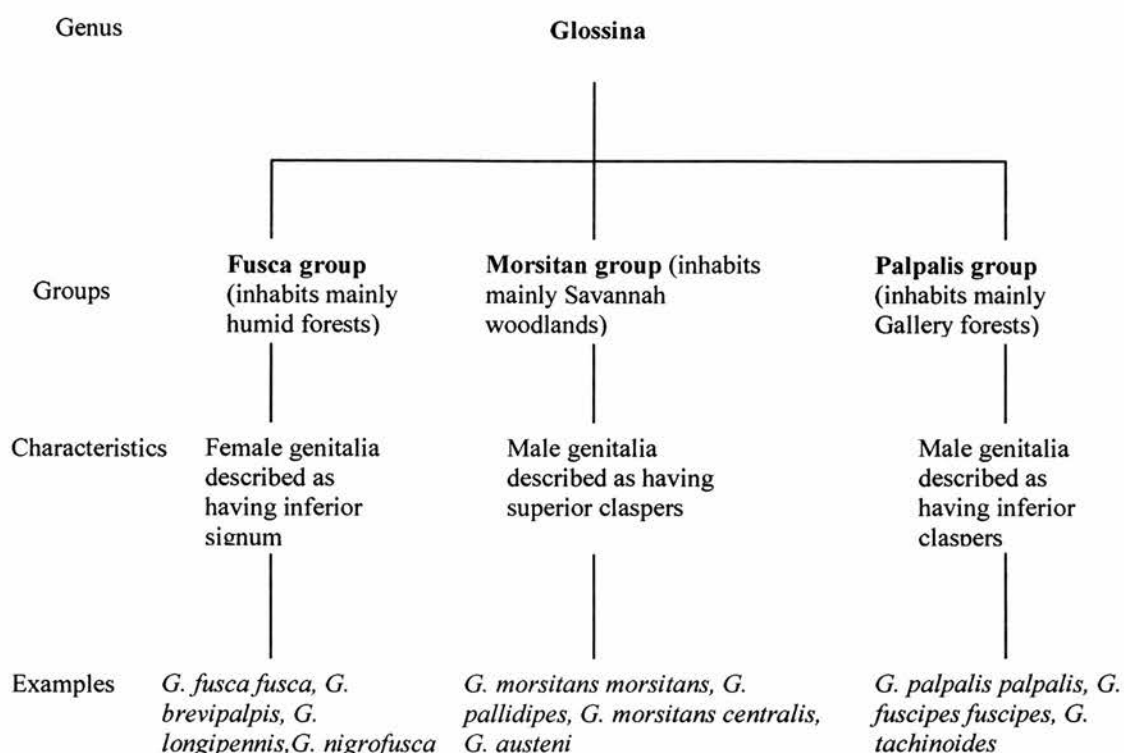


Figure 9: Grouping of the tsetse flies

The *Palpalis* group includes the flies whose male genitalia are characterized as having inferior claspers (Mulligan, 1970). They are distributed in West Africa from the wet mangrove and rain forests of the coastal region northwards into the dry savannah. Members of this group include *G. palpalis palpalis*, *G. palpalis gambiensis*, *G. fuscipes fuscipes*, *G. fuscipes quanzensis*, *G. fuscipes martinii*, *G. tachinoides*, *G. pallicera*, *G. pallicera pallicera* and *G. caliginea*.

### ➤ **The life cycle of tsetse flies**

Tsetse flies undergo a life cycle which includes the eggs that take between 50 to 60 hours to hatch into the 1<sup>st</sup> instar larvae. The 1<sup>st</sup> instar larvae take 1 to 2 days to develop into the 2<sup>nd</sup> instar larvae. These take 2 days to develop into the 3<sup>rd</sup> instar larvae. The eggs and all the larval stages are within the adult female fly. The 3<sup>rd</sup> instar larvae take 2.5 days to develop into pupae. The puparium stage lasts between 30 and 40 days buried underground before emergence into adults. This is also the stage in which spermatogenesis occurs in male flies



(Mulligan, 1970). The adult flies can live for 3 to 4 months, during which they mate and the females lay their pupa (Figure 10).

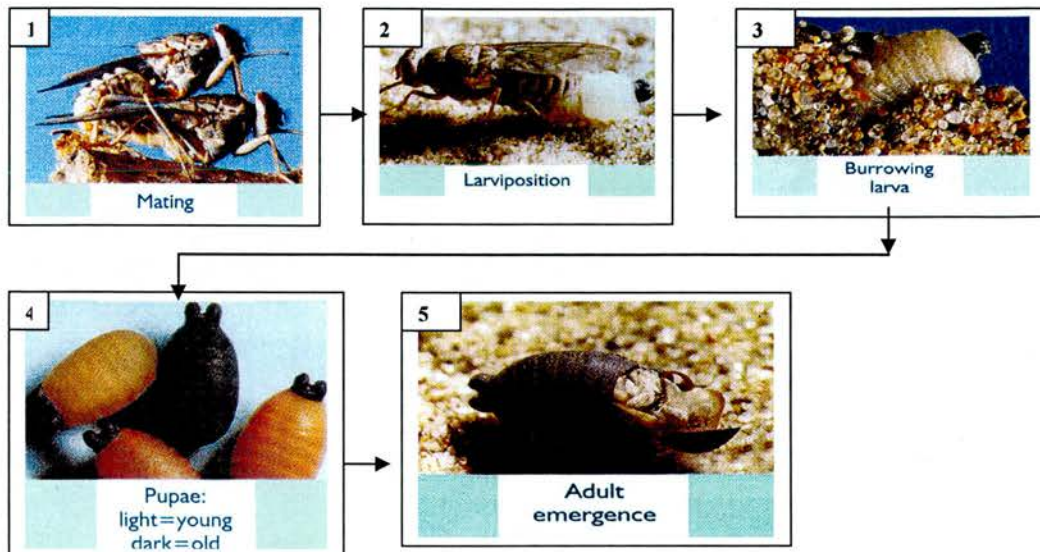


Figure 10: The Life cycle of tsetse fly, after mating (1) the eggs hatch into the 1<sup>st</sup> instar larvae inside the fly which develop upto the 3<sup>rd</sup> instar larvae that is deposited on the ground (2). The larvae then burrow into the soil, (3) and develop into pupae, (4) that eventually develop into the emerging adult, (5). (Adapted from Trypanosomiasis, Tsetse and Africa- The Year 2001 Report, a DFID Publication)

#### 1.4.7.2 The reservoir hosts

In Africa wildlife are considered to be the main, but not necessarily exclusive, reservoir hosts for trypanosomes. It had been established as early as 1965 that cattle can also serve as reservoir hosts especially for human trypanosomiasis, (Onyango, *et al.*, 1969). Recently, it has been shown that domestic animals such as pigs can also serve as reservoir hosts for *Trypanosoma brucei* species, (Kageruka, 1989; Makumyaviri, *et al.*, 1989; Schares and Mehltitz, 1996; Jamonneau, *et al.*, 2003; Waiswa, *et al.*, 2003).

#### 1.4.7.3 African trypanosome infections in livestock

The symptoms and lesions resulting from trypanosomiasis in livestock are for the most part common for infections by all the different parasite species. The lesion, known as a chancre (Apted, 1970), is the result of an inflammatory response, which is characterized by congestion, oedema, and extravasations of polymorphonuclear leukocytes. Plasma cells,

lymphocytes and macrophages accumulate in this lesion and it is from here that the parasites move into the lymphatic and circulatory systems. As the parasitaemia heightens, the body temperature rises to 39 to 41°C. An increased immune response is triggered and hyper-stimulation results in enlargement of the lymph nodes and spleen. In addition, B lymphocyte proliferation leads to an increase in circulating IgM (Luckins and Mehltz, 1976; Whittle, *et al*, 1977). This is associated with the production of antibodies, which are specific to the parasite, host or non-specific. While the host responds aggressively to the parasite invasion, anaemia sets in as the number of erythrocytes and haemoglobin levels rapidly decline and iron consumption goes up (Mamo and Holmes, 1975; Dargie *et al*., 1979a, b; Preston *et al*., 1982b). Anaemia is a significant indicator of disease progression and plays a major role in the pathogenesis of bovine trypanosomiasis.

The disease syndrome is classified as acute or chronic although such categorization is not rigid. For example, acute infections with *T. vivax*, which is characterized by massive haemorrhage, may result in death after 2 weeks, whereas *T. congolense* infections may be fatal by the sixth to tenth week. Chronic disease can be regarded as that where infection persists over 3 months. From inoculation with the pathogen to death or chronic carrier-state, certain pathological changes are evident.

#### **1.4.7.4 The pathology of animal trypanosomiasis**

Trypanosomiasis is a disease whose course and severity depends largely on the species and breed of the animals infected and the type of trypanosome causing the infection (Murray, *et al*, 1982). The disease can be divided into two main categories when examining its pathology: 1) the haematic group, confined to the blood and lymphatics that include infections with *T. vivax* and *T. congolense*; and 2) the humoral group which invade the intercellular spaces in addition to the blood and lymphatics and involve infection with *T. brucei* (Losos and Ikede, 1972).

##### **➤ The haematic group**

The two species involved in this course of pathology cause the greatest economic losses in cattle to the farmers (Williamson, 1970). In experimental infections, the initial response following a tsetse bite is a local skin reaction that manifests in a swelling, 10-30mm in diameter which may turn soft and reddish, referred to as the chancre (Roberts, *et al*, 1969;



Apted, 1970). Initial parasite multiplication has been shown to occur at the dermal collagen and local lymphatic vessels from day 7 to 15 after an infective tsetse bite. This induces acute inflammatory response involving mononuclear cells such as the plasma cells, monocytes, and the lymphocytes (Gray and Luckins, 1980). Prior to the onset of pyrexia and parasitaemia there is a progressive enlargement of the local lymph nodes which is then followed by a series of waves of parasitaemia and onset of anaemia, that is characteristic of the chronic disease or continuous parasitaemia peak.

During acute phase there are fluctuating waves of parasitemia coupled with a massive destruction of erythrocytes due to a hyperplastic mononuclear phagocytic system (MPS) that results in extra vascular haemolysis while postmortem results have shown massive haemorrhages with marked splenomegaly as well as a depleted and pale bone marrow. *Trypanosoma vivax* infections in particular, manifest a wide range of clinical syndromes such as acute haemorrhagic syndrome that is characterized by pyrexia, high parasitaemia, multiple haemorrhages on the mucous and serous surfaces, lymph nodes and a swollen and congested liver. Postmortem results have shown haemorrhages on all organs, with death following within three to four weeks after infection (Losos and Ikede, 1972; Hudson, 1944) on the other hand, the disease may simply present as a chronic low grade infection.

*Trypanosoma congolense* infection is somewhat similar to that of *T. vivax* but virulence ranges from the acute syndrome noticed in *T. vivax* infections to a long chronic phase with severe anemia. The acute phase of the disease caused by *T. congolense* may last for one month after which the animal may recover, die or the disease may progress into a long chronic phase. Postmortem results have revealed the presence of ischaemic lesions in the brain due to the affinity of the parasite for capillary vessels (Losos and Ikede, 1972; Banks, 1979). In both *T. congolense* and *T. vivax* infections, the disease is characterized by progressive emaciation, weight loss and intermittent pyrexia which are associated with low parasitaemia and anaemia (Morrison, *et al.*, 1981). Animals that survive the initial phase of the disease and progress to the second phase have persistent pyrexia, with low and sporadic parasitaemic waves as anemia also progresses to severity. Animals that enter the third phase of the disease remain anaemic, but become mostly aparasitaemic with occasional acute relapses which have been associated with stress due to draught work, malnutrition or pregnancy (Fiennes, 1970). While a small number of animals may recover at this stage, the

majority of the animals tend to succumb to death after 6 to 12 months of infection, or remain stunted in growth, emaciated or infertile. Murray (1974) had shown that during the chronic phase of trypanosomiasis death is caused by congestive cardiac failure due to anemia and circulatory problems associated with increased vascular permeability and myocardial damage.

The main pathological features of animal trypanosomiasis are emaciation and anaemia. While the mechanisms leading to anaemia have not been definitively elucidated (Murray and Dexter, 1988), there is some evidence both from the field and experimental infections that trypanosome infections impose a metabolic stress on infected animals and that the physiological production status, such as lactation, and the level of nutrition have a direct consequence on the animal's ability to tolerate the disease (Agyemang, *et al.*, 1992). It has also been shown that trypanosome infections cause disruption of the lipid metabolism, (Wellde, *et al.*, 1989b; Katunguka-Rwakishaya *et al.*, 1995) and carbohydrate metabolism in ruminants (Wellde, *et al.*, 1989b), the two critical factors which may help explain emaciation in animal trypanosomiasis (Figure 11). However, this may be a common feature for all trypanosome infections since the chronic human infections also show emaciation as is evident (Figure 12).

Anemia has been shown to occur in two distinct phases: the first phase which is associated with the first wave of parasitaemia is thought to be caused by extra-vascular haemolysis due to an expanded mononuclear phagocytic system (Morrison, *et al.*, 1981; Murray and Dexter, 1988; Jennings, *et al.*, 1974; Mamo and Holmes, 1975). The mechanism for this type of anemia is likely to involve trypanosome hemolysis, complement system activation and immune complexes (Murray and Dexter, 1988). Although erythropoiesis is usually elevated at this stage, the effect is not adequate to compensate for the massive loss (Dargie, *et al.*, 1979; Katunguka-Rwakishaya, *et al.*, 1992). This type of anemia is usually described as normochromic and normocytic although the role of hemodilution caused by an expanded plasma volume is unknown (Mamo and Holmes, 1975; Dargie, *et al.*, 1979b). In the second phase of the disease the high rate of red cell destruction continues even though there may not be any detectable parasites in circulation while the bone marrow shows dyshemopoiesis possibly due to a defect in iron metabolism (Murray and Dexter, 1988; Dargie, *et al.*, 1979).

Other possible factors involved in causing anaemia include haemolysins produced by the parasite, fever and immunological factors.

➤ ***The humoral group***

In the early stages of infection with *T. brucei* species the spleen and lymph nodes are enlarged with oedematous skin plaques and serous effusion into the peritoneum and pericardial cavities (Lossos and Ikede, 1972), but the most affected organs are the heart and the brain (Morrison, *et al.*, 1983). Cattle that developed clinical symptoms of CNS invasion were found to have a diffuse meningo-encephalitis with marked cellular invasion of the perivascular areas, meninges and choroid plexus mainly by the plasma cells and lymphocytes. In their study, Morrison *et al.*, (1983) reported infections in cattle with *T.b. brucei*, although they found that only some stocks of *T. b. brucei* were able to produce a marked anemia and occasional mortality. Experimental infection of cattle with *Trypanosoma brucei rhodesiense* had shown the development of the disease similar to sleeping sickness in man with involvement of the central nervous system, (Wellde, *et al.*, 1989b). This study also showed that the animals that developed a CNS syndrome became anaemic and that the disease was indeed fatal to cattle, with the development of parasitaemia as well as detectable parasites in the lymph nodes and in the CSF. In both of these studies, the animals were given needle injections of parasites.

Natural infections of cattle with *T. brucei* have been reported in West Africa (Godfrey and Killick-Kendrick, 1960) and in East Africa (Wellde, *et al.*, 1989c). These infections have not been considered to be of any serious pathogenicity since they were marked by low parasitaemia and mild anemia and are generally thought not to cause any serious disease, (Hornby, 1921; Killick-Kendrick, 1971). However, during an outbreak of sleeping sickness in the Lambwe Valley region in Kenya in 1980, it was reported that the mortality rate of cattle due to *T. b. brucei* infections went up to 38% (Wellde, *et al.*, 1989c).



Figure 11: A chronic stage of infection with *T. congolense* in a cow, (Courtesy ILRI Graphics)

#### 1.4.7.5 Trypanosome infections in sheep and goats

Sheep and goats are important in many countries of sub-Saharan Africa (Winrock International, 1992) where tsetse transmitted trypanosomiasis is a major disease constraint to small ruminant production. However, for many years it was believed that due to their agility, fecundity and tolerance of trypanosomal infections, sheep and goats were least affected by trypanosomes (MacLennan, 1970). These animals have however been shown to acquire trypanosomal infections (Griffin, 1978). Experimental infections result in serious pathology associated with severe clinical signs such as raised temperature, increased metabolic rate and reduced feed intake (Zwart, *et al.*, 1991). The pathology of trypanosomiasis in sheep and goats is generally similar to that of cattle.

#### 1.4.7.6 Trypanosome infections in pigs

Pigs are generally thought to be refractory to *T. vivax* infections. However, recent studies have shown that pigs indeed can carry *T. brucei* infections and act as reservoirs of infection (Kageruka, 1989; Makumyaviri, *et al.*, 1989; Schares and Mehlitz, 1996; Jamonneau, *et al.*, 2003; Waiswa, *et al.*, 2003). Experimental infection of pigs with *T. brucei* produces a severe disease that results in clinical disturbances of the CNS, (Otesile, *et al.*, 1991).



Natural infections with *T. brucei* are generally considered to be of low pathogenicity, making them ideal as reservoir hosts (Waiswa, *et al.*, 2003). *T. congolense* does affect pigs but with low pathogenicity, however, *T. simiae* is the most pathogenic species that affects pigs, causing massive parasitaemia and death after four to six days of infection (Stephen, 1966).

#### **1.4.7.7 Trypanosome infections in dogs and cats**

Dogs and cats are generally susceptible to *T. b. brucei* infections, where an acute form of the disease is evident and death results within two months after infection. Infections of dogs with *T. b. rhodesiense* also result in an acute disease while infections with *T.b. gambiense* result in a chronic form of the disease (Losos and Ikede, 1972). *T. congolense* can also cause an acute form of the disease in dogs. However, these animals are generally refractory to *T. vivax* and *T. simiae* infections, (Jordan, 1986).

#### **1.4.7.8 Trypanosome infections in camels**

Camels can be infected by *T.b. brucei*, *T. congolense*, *T. simiae* and *T. viva*. where the disease can be either acute or chronic, with symptoms similar to those of other domestic animals. However, *T. evansi*, the main parasite responsible for camel trypanosomiasis, which is spread by mechanical transmission, occurs in the areas away from the tsetse belt such as the Middle East, Asia and parts of north Africa.

#### **1.4.7.9 Trypanosome infections in humans**

Human trypanosomiasis (sleeping sickness) has been in existence in Africa for many centuries but the disease was not described until the 1800s and 1900s by the European settlers and the colonialists (Hide, 1999). Three severe epidemics have been observed in Africa in the last century. The 1896-1906 epidemic which was confined to Uganda and the Congo basin with an estimated 1 million human lives lost; the 1920s epidemic which occurred in several African countries and finally the third and on-going epidemic which started as a result of the re-emergence of the disease in the 1970s, (Ford, 1971). Humans can only be infected by two sub-species of *Trypanozoon*: namely *Trypanosoma brucei rhodesiense* and *T. b. gambiense* the former is commonly found in Eastern Africa while the latter is more prevalent in West Africa. Sleeping sickness due to *T.b. rhodesiense* covers areas including western Kenya, SE Uganda, Rwanda, Burundi, Tanzania, Mozambique,

Zambia, Malawi, Zimbabwe, Botswana and Angola, an area approximately 2.72 million square kilometres inhabited by more than 50 million people.

The common pathological features of human African trypanosomiasis are wasting of body condition, Figure 12, marked by cerebral oedema and non-purulent meningitis, bronchopneumonia and enlargement of both the spleen and liver.

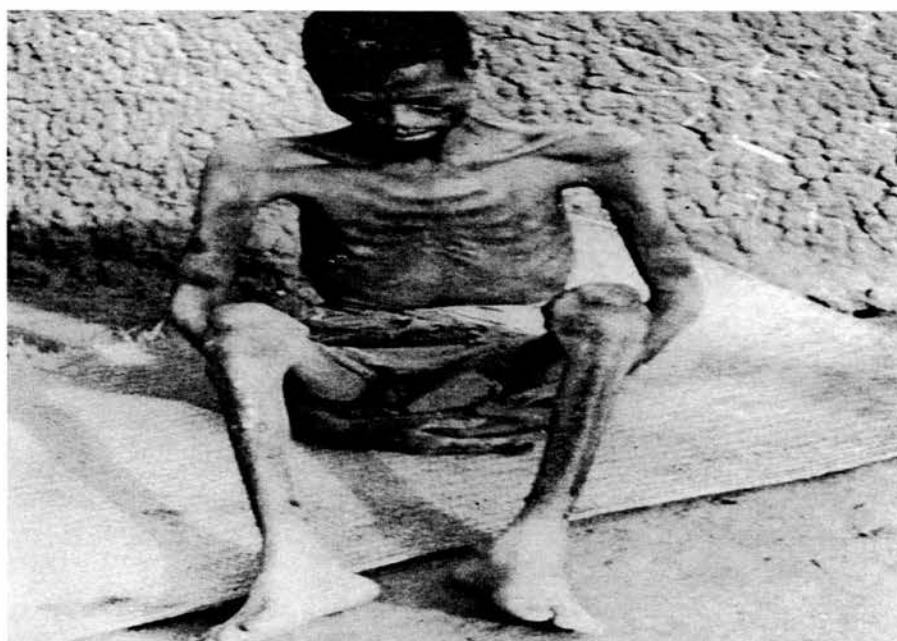


Figure 12: A chronic case of sleeping sickness (human African trypanosomiasis, (HAT))

#### **1.4.8 Control of trypanosomiasis**

The control of trypanosomiasis currently relies upon the vector control and treatment of the infected host with either prophylactic or curative drugs.

##### **1.4.8.1 Vector control**

A variety of methods have been used in attempts to reduce tsetse fly populations. These methods include the use of insecticides applied on odour-baited traps or screens or applied as aerosols by air or ground spraying (MacLennan, 1981). Cattle dipping or pour-on are

other target method used to kill tsetse. Insecticides like deltamethrin, which is used as a wash or pour on, are used where up to 100% of alighting flies can be killed within two weeks. However, for the technique to succeed, domestic livestock must be present in the area in sufficient numbers, cattle must represent the overwhelming proportion of the host complex of the tsetse area and most of the cattle have to be presented for treatment on regular basis. Unfortunately, the cost of the insecticides is a serious constraint for this method to be sustained (Chadenga, 1994). In recent years, the use of insecticides has lost favour among tsetse control institutions due to environmental concerns regarding the use of DDT, dieldrin and other insecticides. Treated areas are easily reinvaded necessitating costly re-treatment programmes. Furthermore, these methods do not immediately protect cattle from being infected with trypanosomes.

Theoretically, the low reproductive capacity and infrequent mating of the female tsetse flies makes them ideal candidates for control by genetic techniques. One of these techniques - the sterile insect technique (SIT), involves the release of male flies that have been sterilized with gamma irradiation into the environment to compete with the wild males in mating. This method has been used successfully in Zanzibar where *G. austeni* males sterilized by gamma irradiation were dispersed by air over the whole island in 1994. No wild tsetse have been caught since September 1996 and the eradication of *G. austeni* from Zanzibar was declared at the end of 1997 (Saleh *et al.*, 1998). However, this method has problems which include firstly the enormous cost of implementation of the program. Secondly, the density of the target population must be low for SIT to be effective (Politzar and Cuisane, 1982). Thirdly, this method works best in an isolated area like an island, as evidenced in Zanzibar in order to avoid re-invasion by new migrant flies.

Other methods that have been used in the control of the tsetse vector include bush clearing, elimination of wild animal reservoir hosts and biological control. Bush clearing renders the habitat unsuitable for tsetse habitation, but this method has disadvantages, which include decrease in soil fertility, soil erosion and adverse effects on water supplies. Elimination of wild animal hosts is not a popular method as African economies rely on tourism as a source of foreign income and wild animals are among the major attractions for tourists.



Biological control involves the use of natural enemies of the tsetse vector such as parasites and predators like fungi, bacteria and parasitic mites. To be successful, biological control organisms generally have to originate from a different geographical or ecological area from the potential pest to be controlled otherwise it would be expected that the control and target organisms would be sufficiently adapted to one another that no significant degree of control would easily be maintained over a long period. Most trials of parasites on tsetse flies have used insects that occur naturally in tsetse habitats, which may partly explain the lack of success.

Entomopathogenic fungi and bacteria have been used in experimental studies to show that they could be potential candidates for the control of trypanosomiasis. These include *Pseudomonas aeruginosa*, *Serratia marcescens* and *Bacillus sphaericus*, (Kaaya and Darji, 1989). The advantages of using biological control methods include the fact that they do not affect the environment negatively and it is a self-sustaining method. Once the entomopathogens are introduced in the environment, they will multiply and attack their intended targets naturally. However, these methods have shortfalls such as the difficulty in choosing a suitable method of formulation and dispersal of the pathogens and the chances of adaptation of the insect target to the pathogen so that they end up having a symbiotic relationship rather than a pathogen-host relationship (Kaaya, 1989).

#### **1.4.8.2 The use of chemotherapeutic and chemoprophylactic drugs in the control of trypanosomiasis**

The use of drugs for the prevention and treatment of trypanosomiasis has been practised for many decades (Leach and Roberts, 1981). However, the rapidity with which the trypanosomes are developing resistance to the drugs has complicated this approach to controlling the disease. In spite of this, some of the older chemoprophylactic drugs such as the quinapyramine derivatives Antrycide and Antrycide Prosalt are still used and give effective protection against *T. b. brucei* infection in horses, camels, and cattle for up to 3 months. The drug pyrimethidium bromide (Prothidium and AD2801) is useful in the prophylaxis of *T. vivax* and *T. congolense* infections in cattle, sheep, and goats and can give protection for up to 6 months.

The most widely used chemoprophylactic drug which is also the least expensive is isometamidium chloride (Eisler, 1996). This drug which has been in use for over 20 years and sold under the trade names Samorin, Trypanidium, and M&B 4180A, gives protection for 3-6 months against all the three major African animal trypanosome species. Isometamidium is also used as chemotherapeutic agent along with the other quaternary ammonium trypanocides Antrycide, Ethidium and Prothidium. Homidium bromide has also been found to be an effective chemoprophylactic drug in Kenya, and the newly introduced arsenical Cymelarsan is effective in the treatment of *T. b. brucei* infection. A very widely used chemotherapeutic drug is diminazine aceturate (Berenil<sup>®</sup>, Hoechst, Germany), which is effective against all three African animal trypanosomes. However, the development of resistance to these drugs has been reported in both East and West Africa (Rottcher and Schillinger, 1985; Schonefeld *et al.*, 1987; Mulugeta, *et al.*, 1997; McDermott, *et al.*, 2003; Eisler, *et al.*, 2003; Shinyangwe, *et al.*, 2004). Furthermore, although extensively used in trypanosomiasis control, chemoprophylaxis is expensive and time-consuming and thus unsustainable as a long-term solution to the problem of African animal trypanosomiasis.

#### **1.4.8.3 Immunological control of the disease**

The control of African trypanosomiasis is still a big problem as new disease control strategies which include the use of vaccines have not yet been achieved although this is a most desirable option. However, an attempt to develop a vaccine against the disease has been thwarted by the parasite's ability to change the composition of its exposed surface antigens (Cross, 2004). Since conventional approaches to the control of the disease have largely met with failure, there has been a renewed interest in identifying novel aspects of the biology, biochemistry and molecular biology of trypanosomes that might be exploited to develop new targets for vaccines. If developed, then vaccination could form the best option for the control of this disease (Hajduk, *et al.*, 1992).

#### **➤ The trypanosome variable surface glycoprotein (VSG)**

The surface of both the metacyclic trypanosomes that enter the mammal during a tsetse fly meal and the subsequent proliferating bloodstream forms are covered with a mono-layer of a variable surface glycoprotein (VSG). The VSG forms a barrier between conserved proteins in the cell membrane and the effector molecules of the host immune system (Sayed *et al.*, 2000). Once host antibodies that recognize any one VSG reach a high enough titre the

trypanosomes expressing that particular VSG are killed. The persistence of an infection, despite the host mounting an immune response, is dependent on antigenic variation of the VSG (Cross, 1978), which occurs through stochastic changes in which a single *VSG* gene is expressed from a repertoire of possibly a thousand. The switch in VSG expression occurs either through gene conversion at the active VSG expression site or through inactivation of one VSG expression site and activation of one of the ~20 other VSG expression sites.

#### **1.4.8.4 The use of trypanotolerant animals**

Studies have shown that some indigenous breeds of cattle in West Africa such as the N'Dama have a significant degree of trypanotolerance and are able to thrive in tsetse-infested areas where susceptible breeds cannot survive (Shaw & Hoste, 1987). The raising of such trypanotolerant animals is one other method that can be used in the control of trypanosomiasis in livestock and such cattle have been imported into countries such as Gabon and Democratic Republic of Congo, where they are successfully being raised on several large ranches (Shaw & Hoste, 1987). Although trypanotolerant cattle offer considerable potential as an alternative means of raising cattle in tsetse-infested areas, they currently represent only 5% of the cattle population of sub-Saharan Africa. Furthermore, due to the limited availability of animals of these breeds and because of their small size they are not always popular with farmers who aspire to owning the larger improved breeds. For these reasons their use in wide-scale programmes to restock tsetse-infested areas is limited.

### **1.5 DIAGNOSIS OF AFRICAN TRYPANOSOMIASIS**

#### **1.5.1 Clinical diagnosis**

In both *T. congolense* and *T. vivax* infections, the disease is characterized by progressive emaciation and weight loss, intermittent pyrexia associated with low parasitaemia and anemia (Morrison, *et al.*, 1981). Animals that survive the initial phase of the disease and progress to the second phase have persistent pyrexia, with low and sporadic parasitaemic waves as anemia also progresses to severity. These features mark the general basis of clinical diagnosis of trypanosomiasis

### 1.5.2. Laboratory diagnosis

Laboratory diagnosis of the disease has been the best way of determining if the clinical symptoms presented by an animal are due to trypanosome infection. This involves the examination of blood, the cerebro-spinal fluid (CSF) or the lymphatic fluid for the presence of parasites or parasite molecules or complexes of the host-parasite interaction. Over the years a number of techniques have been developed for the diagnosis of both human and animal trypanosomiasis that include the standard trypanosome diagnostic techniques (Wilson, 1969).

Parasitological diagnostic techniques are designed to demonstrate the presence of parasites in the blood, CSF and lymphatic fluid through microscopic examination and include the examination of wet, thin and thick blood films. Wet films have the advantage of being fast and simple to carry out while giving some indication of the species of parasites present due to the characteristic mobility of some trypanosome species. However, the sensitivity of this method is quite low; low levels of parasitaemia cannot be detected with this method. Thin and thick smears normally stained with Romanowsky stains such as Giemsa, May-Grunwald or Diff-Quick stains offer better species characterization since morphological studies can be done more carefully under higher magnification; however, they are less sensitive in detecting low parasitaemia.

A number of concentration techniques have been developed to demonstrate the presence of trypanosomes in the host animal blood under the microscope. These include the haematocrit centrifugation technique, (HCT) (Woo, 1970); the capillary concentration technique, (CCT) (Walker, 1972); the miniature anion-exchange/centrifugation technique (m-AEC) (Lumsden, 1979) the hypotonic lysis method, (HLM) (Leefflang, *et al.*, 1974) and the dark ground phase contrast buffy coat technique (DG) (Murray, *et al.*, 1977). The sensitivities of these techniques were compared and evaluated by Paris, *et al.*, (1982). Apart from being somewhat more sensitive, some of these concentration techniques also allow for the packed red cell volume (PCV) or haematocrit estimation thus giving immediate indication of the state of anemia.

### 1.5.3 Xenodiagnosis

This technique is of significant value where trypanosomes in the peripheral blood vessels are in such low levels that cannot be detected by any of the above mentioned techniques. Susceptible laboratory mice strains are normally injected with the host blood and the development of the parasites is monitored as they multiply in the mice. However, the technique depends on the susceptibility of the mice to the infecting parasite since not all trypanosome species can infect rodents. Moreover, the technique takes longer to yield results and requires elaborate and expensive maintenance for the animals.

### 1.5.4 Serological diagnosis

A number of serological diagnostic tests have been developed which demonstrate the antibodies against trypanosomes present in the serum of infected animal. However these tests tend to give cross reactions to other protozoan parasites or across species when there are mixed trypanosome infections in the animal, thus resulting in non-specificity. They may only indicate that the animal might have had some exposure to trypanosomes in the past, (Luckins, 1977). These methods include the complement fixation test (CFT) that is used in the diagnosis of *T. equiperdum*; indirect fluorescent antibody test (IFAT), the most widely used serodiagnostic test against animal trypanosomiasis, and found to be more sensitive than all the STDM, (Molineux, 1975; Wilson, 1969; Ashkar and Ochilo, 1972); card latex agglutination test, (CATT), which has been widely used in West Africa for the diagnosis of *T.b. gambiense* infections in humans (Magnus and van Miervenne, 1978); CAAT<sup>®</sup>, Brentec Diagnostics Ltd, Nairobi, Kenya (Olaho-Mukani and Nyang'ao, 1993) enzyme linked immunosorbent assay (ELISA) which may be either for antibody or antigen detection (Engvall and Perlman, 1972; Voller, *et al.*, 1975; Luckins, 1977; Nantulya and Lindqvist, 1989). These tests have been found to be more sensitive than most of the parasitological diagnostic techniques. However, these serological diagnostic tests cannot distinguish between past and present infection after treatment and whether the parasite itself is present or absent, or differentiate between infections with multiple species due to less or no cross reactivity.



### **1.5.5 Molecular based diagnostic techniques for trypanosomes**

Normally the number of trypanosomes in the blood of infected livestock is inherently lower than many parasitological techniques are capable of diagnosing. Moreover, antibody detection techniques do not clarify whether the infection is past or current and they are not yet sufficiently reliable. Over the years a number of molecular based techniques have been developed that allow for the detection of unique trypanosome species and strains. These include isoenzyme characterization (Gibson, *et al.*, 1980; Tait, 1980); restriction fragment length polymorphism (RFLP) (Hide, *et al.*, 1991); polymerase chain reaction (PCR) amplification based techniques using minisatellite markers (MacLeod, *et al.*, 2000); RAPD (Mathieu-Daude, *et al.*, 1995); AFLP (Masiga, *et al.*, 2000); mobile genetic element (MGE)-PCR (Hide and Tilley, 2001); serum resistance associated (SRA) gene-PCR (Welburn, *et al.*, 2001; Gibson, *et al.*, 2002).

#### **1.5.5.1 The polymerase chain reaction (PCR) amplification**

PCR amplification, using primer-directed enzymatic amplification with a thermo-stable *Thermus aquaticus* DNA polymerase (Saiki, *et al.*, 1988), employs molecular markers specific to individual species and/ or sub-species and provides the dual advantages in the improved sensitivity as well as specificity (Artama, *et al.*, 1992; Masiga, *et al.*, 1992; Majiwa, *et al.*, 1994; Masake, *et al.*, 1994; de Almeida, *et al.*, 1998; Kabiri, *et al.*, 1999; Desquesnes, *et al.*, 2001; Penchenier, *et al.*, 2000; Welburn, *et al.*, 2001). This technique offers better opportunity to overcome the insufficiencies of both parasitological and serological methods of trypanosome detection in both the vector and animal host. There are now an increasing number of publications which report the practical and wider field applications of PCR for epidemiological studies of trypanosomiasis both at the vector and the animal host level, (de Almeida, *et al.*, 1998; Wuyts, *et al.*, 1995; Desquesnes and Tresse, 1996; Kanmogne, *et al.*, 1996; Penchenier, *et al.*, 1996; Masake, *et al.*, 1997; Duvallet, *et al.*, 1999 and Picozzi, *et al.*, 2002). Large-scale surveys of animals using this technique are now possible by sampling the blood or concentrated buffy coat onto a filter paper which is subsequently dried and processed for PCR amplification. The technique has undergone several evaluations for field diagnosis of cattle trypanosomiasis (Katakura, *et al.*, 1997). PCR has been used to detect *T. vivax* in goats (de Almeida, *et al.*, 1997; 1998); *T. congolense* in cattle (Masake, *et al.*, 1997) and trypanosomes in the mouth parts of tsetse flies (Majiwa, *et al.*, 1994; Duvallet, *et al.*, 1999; Morlais *et al.*, 2001).

## 1.6 EPIDEMIOLOGY OF TRYPANOSOMIASIS IN UGANDA

In Uganda, trypanosomiasis constitutes a major health problem that affects both man and livestock. For many years sleeping sickness (SS), has occurred in both south eastern Uganda and north and north western Uganda, covering Soroti, Kamuli, Iganga, Mayuge, Bugiri, Mbale, Tororo and Busia districts (Figure 13) at times in epidemic proportions. In south eastern Uganda, the acute form of sleeping sickness puts at risk a human population of approximately 3.3 million people, whilst animal trypanosomiasis puts at risk about 2.8 million livestock. The prevalence of the animal disease is up to 40% in some areas, (FITCA, 2001).

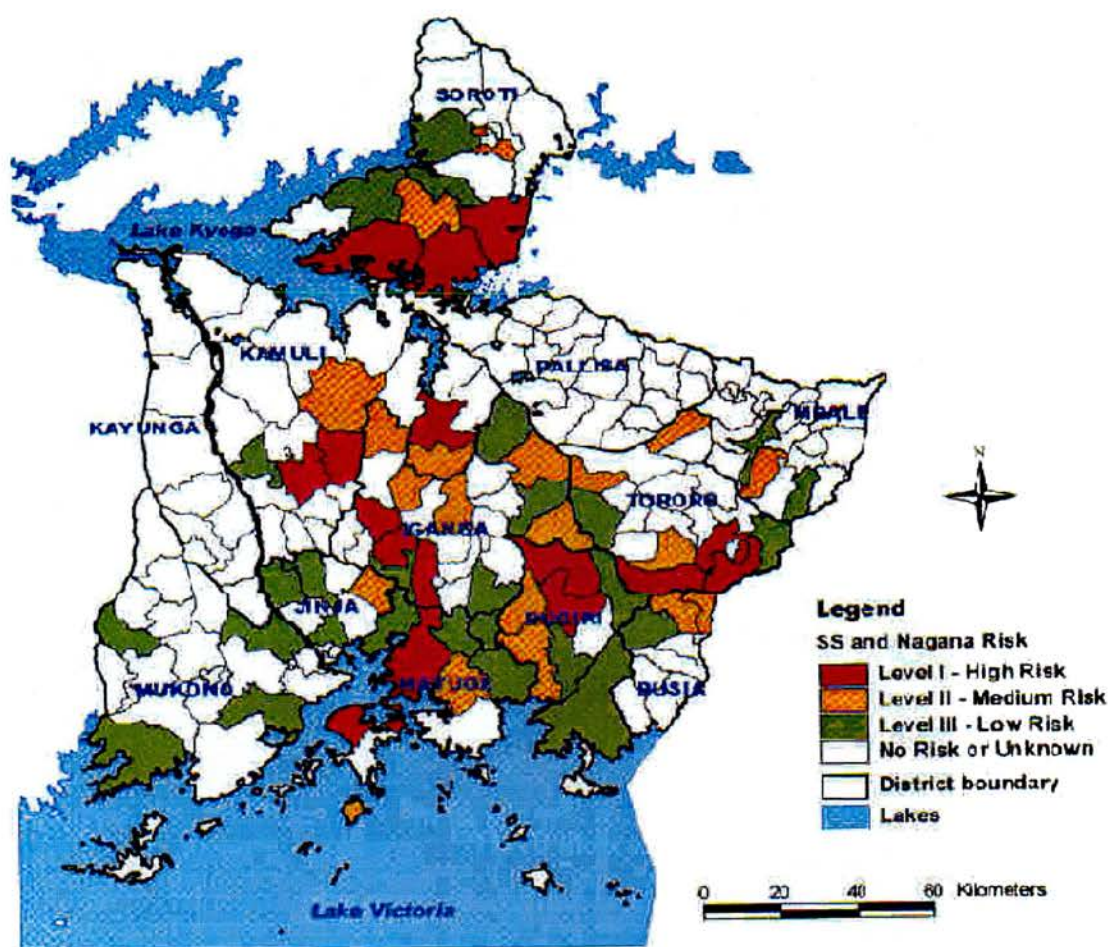


Figure 13: Geographical locations of the sleeping sickness and nagana risk areas in Uganda, (FITCA -Uganda, 2003)



In nearly 100 years no sustainable progress has been made in tsetse and trypanosomiasis control in Uganda. This is largely due to civil strife, socio-economic problems and the fact that in the past tsetse control strategies have relied on sophisticated technologies such as aerial spraying, which are inappropriate to Uganda's economic and environmental situation, (Okoth, 1999). The fly vector for sleeping sickness in Uganda, *G. f. fuscipes*, has been observed to be resting in *Lantana*, coffee and banana plants thus it became peri-domestic, a factor that has led to the transmission cycle being domestic too, that is, animal-fly-man, (Abaru, 1989). It has been suggested that community participation using appropriate technologies such as low-cost traps/targets and integrating farming activity with tsetse control seem to be the most appropriate approach in SE Uganda, (Okoth, 1999).

## **1.7. TICK-BORNE DISEASES (TBD) OF RUMINANT LIVESTOCK**

The most important tick-borne diseases of economic importance are caused by the rickettsial organisms *Anaplasma marginale* and *Cowdria ruminantium*; *Babesia bovis*, *B. bigemina*, *Theileria annulata*, and *T. parva* (Young, Grocock and Kariuki, 1988; Sewell and Brocklesby, 1990; Uilenberg, 1995; de Castro, 1997). In 1984 the Food and Agriculture Organization (FAO) estimated that the global annual losses due to ticks and TBD was approximately US\$7 billion (Uilenberg, 1995) while current estimates put the losses at between US\$ 13.9 and 18.7 billion (de Castro, 1997). The control of TBD is therefore essential to the improvement of livestock production.

### **1.7.1 The genus *Theileria***

*Theileri*, (Figure 14) are tick-transmitted, obligate intracellular, haemoprotozoan parasites of the phylum Apicomplexa, which include diverse, economically and medically important parasites of vertebrates. The diseases caused by *Theileria* are collectively known as theilerioses. The severity of the disease can vary greatly according to the species and strain of parasite, the dose of parasites infecting the host, and host susceptibility (Irvin and Morrison, 1985). The most severe theilerioses are lymphoproliferative disorders which may be associated with leucopenia and/or anaemia (Irvin and Morrison, 1985). *Theileria* infect livestock, including cattle, goats, sheep and horses (and possibly camels) throughout much of the world, but the most pathogenic and economically important species are found in the tropics and sub-tropics (Uilenberg, 1995). Wild or feral bovids and equids are commonly

infected by *Theileria* species, providing important wildlife reservoirs of the parasites, but theileriosis rarely occurs in these animals. A carrier state often occurs in livestock and wildlife infected with *Theileria* (Brown, Hunter and Luckins, 1990).

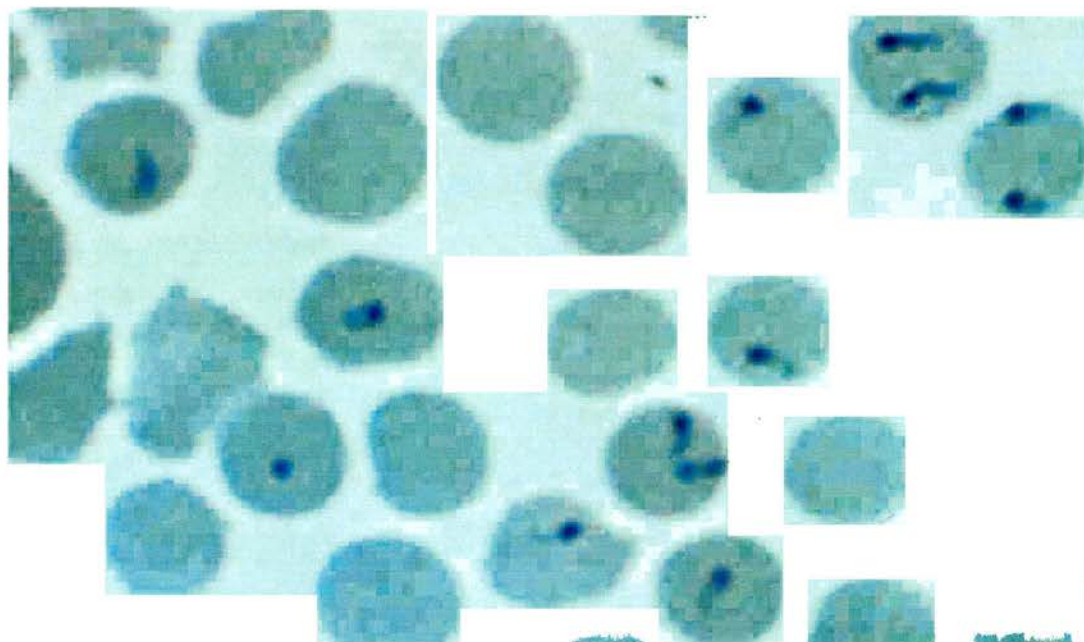


Figure 14: *Theileria parva* parasites in the bovine red blood cells, (Johannes Kaufmann, 1996).

The most pathogenic *Theileria* species that infect cattle are *T. parva* and *T. annulata*, while sheep can be infected by *T. lestoquardi* (Uilenberg, 1981). *T. mutans*, *T. taurotragi*, *T. velifera*, *T. ovis*, *T. separata* and species of the *T. buffeli/sergenti/orientalis* complex are either apathogenic or generally of low to medium pathogenicity to domestic ruminants. However, the occurrence of these species can confuse the epidemiology of pathogenic species. The species *Babesia equi* is a pathogenic parasite of horses that has many characteristics of *Theileria* and was recently reclassified as *Theileria equi* (Mehlhorn and Schein, 1998).

The taxonomy of the genus *Theileria* (Table 4) has had a long and confusing history, due mainly to the question of whether or not a sexual cycle occurs in the tick vector. The situation has been simplified following the confirmation of a sexual cycle (Mehlhorn and

Schein, 1984) and has been reviewed by Norval, *et al.*, (1992). The current understanding of the taxonomic status of *Theileria* is presented in Table 4.

#### **1.7.1.1 *Theileria parva* (Theiler, 1904)**

*Theileria parva* is a parasite of cattle, African (Cape) buffalo (*Syncerus caffer*) and probably waterbuck (*Kobus* spp.) (Stagg, *et al.*, 1994). Reports of *T. parva* causing mortality in buffalo are rare (Neitz, 1957; Norval, *et al.*, 1992), although in cattle, especially productive taurine breeds, *T. parva* causes a severe and often fatal lymphoid disease known as East Coast fever (ECF). ECF occurs in parts of eastern, central and southern Africa where conditions are suitable for the main tick vector, *Rhipicephalus appendiculatus* (Norval, *et al.*, 1992), in equatorial East and southern Africa. Other vectors include *R. duttoni*, (Angolan plateau); *R. zambeziensis*; *Hyalomma excavatum*; *H. dromedarii* and *H. truncatum*. In 1989, direct and indirect cattle production losses due to ECF were estimated at \$169 million (Mukhebi *et al.*, 1989). In Uganda, the disease known locally as ‘Amakebe’, is often seen in calves but rarely in adult indigenous cattle. However, in the exotic breeds the disease affects both calves and adults with high mortality rate, and it has been suggested that ECF be considered in the differential diagnosis of all cases of long-standing illnesses of cattle in the areas under study, regardless of whether there are any tangible clinical presentations (Shannon, 1977).

ECF begins 5-10 days after the infective tick bite and is characterized by high fever, enlargement of the lymph nodes, especially those draining the head, severe pulmonary oedema and wasting. Death usually occurs within three weeks after infection. A lymphoproliferative phase characterizes the early stages of ECF as parasitized lymphocytes undergo rapid clonal proliferation and infiltrate both lymphoid and non-lymphoid tissues of the host, usually within fourteen days of infection. *T. parva*- infected cells have a metastatic behaviour, invading and proliferating in various tissues (Irvin *et al.* 1975; Morrison *et al.* 1981). The major pathology of ECF is caused by the invasion of tissues with parasitized cells, lysis of infected lymphoid cells by cytotoxic T-lymphocytes (CTL) and also extensive major histocompatibility complex (MHC)-unrestricted lymphocytolysis associated with non-specific natural killer cells (Morrison, Taracha and McKeever, 1995; Ahmed and Mehlhorn, 1999; McKeever *et al.* 1999). Although high levels of piroplasm parasitaemia can occur with *T. parva* infection, anaemia and jaundice rarely develop (Irvin and

Morrison, 1985). Previous trinomial sub-speciation of *T. parva* (*T. p. parva*, *T. p. bovis* and *T. p. lawrencei*), which was based on distinct forms of theileriosis, was abandoned because there was no biological justification for the system and it has been recommended that *T. parva* be described as either cattle- or buffalo-derived parasites (Anon, 1989a).

Table 4: Classification and features of *Theileria*, (Levine *et al.*, 1980; Corliss, 1984 & 1986; Irvin, 1987).

Kingdom	Protista	'Lower' eukaryotes
Subkingdom	Protozoa	Single celled eukaryotes
Phylum	Apicomplexa	Apical complex present in at least some stages; reproduce sexually by syngamy; all parasitic
Class	Sporozoea	Sporogonic stage producing sporozoites
Subclass	Piroplasmia	Piroform (pear shaped), rod shaped or amoeboid; parasites in erythrocytes and some other cells
Order	Piroplasmida	Asexual and sexual reproduction. Ticks are vectors. Comprising mainly two genera: <i>Theileria</i> and <i>Babesia</i>
Family	Theileriidae	Schizont stages in lymphocytes
Genus	<i>Theileria</i>	Piroplasm stage in erythrocytes lacks pigment

#### 1.7.1.2 *Theileria mutans* (Theiler, 1906)

*T. mutans* is a parasite of cattle and African buffalo and is transmitted by *Amblyomma* spp, (*Amblyomma variegatum* and *A. hebraeum*). (Uilenberg, *et al.*, 1974; Norval, *et al.*, 1992). It is very widespread throughout sub-Saharan Africa where the distribution follows that of its tick vectors (Walker and Olwage, 1987). Compared to *T. parva* or *T. annulata*, *T. mutans* is generally regarded as non-pathogenic or mildly pathogenic to cattle, including exotic breeds, although there are records of *T. mutans* being pathogenic and even fatal in East Africa (Irvin *et al.* 1972; Snodgrass *et al.*, 1972; Uilenberg *et al.*, 1977; Young *et al.*, 1978b; Mutugi, 1987; Morzaria *et al.*, 1989). The disease is important in cattle imported into endemic areas where an acute form may develop.

The disease caused by *T. mutans* is characterized by malaise, pyrexia and slight swelling of lymph nodes. Anaemia, which is a major clinical sign, can become severe in cases of pathogenic strains (Brown, Hunter and Luckins, 1990). *T. mutans* undergoes rapid,



transient replication of the schizont stage and limited lymphocytic merogony. Its main mode of replication occurs by intra-erythrocytic merogony, which in cases of pathogenic strains results in a high piroplasmic parasitaemia (Norval, Perry and Young, 1992). The degree of anaemia has been shown to correlate with the level of parasitaemia (Young *et al.* 1978b; Paling, Grootenhuis and Young, 1981; Mutugi, 1987; Flach *et al.* 1989).

*T. mutans* can result in productivity losses in infected cattle, especially when present as a concurrent infection with other tick-borne parasites or when the animals are stressed (Paling, Grootenhuis and Young, 1981; Morzaria *et al.* 1981; Saidu, 1981; Mutugi, 1987; Morzaria, 1989; Flach *et al.* 1989; Biwi, Rabia and Dolan, 1992). However, infection by *T. mutans* is only important as a complication during ECF immunization, causing problems when interpreting the results of immunization trials (Kavishe, 1989; Morzaria, 1989; Morzaria *et al.* 1990a; Flach *et al.* 1989; Rabia *et al.* 1993). Buffaloes may serve as reservoirs of *T. mutans* strains that are pathogenic to cattle (Grootenhuis and Young, 1981; Paling, Grootenhuis and Young, 1981; Moll, Lohding and Young, 1984). The precise prevalence of pathogenic *T. mutans* is unknown, due in part to the lack of specific and simple diagnostic tools.

#### **1.7.1.3 *Theileria taurotragi* (Martin and Brocklesby, 1960)**

*T. taurotragi* is a parasite of eland (*Taurotragi oryx*) in which it is occasionally pathogenic and even fatal (Brocklesby, 1962; Grootenhuis *et al.* 1980). It is also infective to cattle, but reactions in cattle are normally subclinical or mild (Grootenhuis *et al.* 1979), although Uilenberg (1981) reported two cases in which severe but temporary clinical reactions occurred. *T. taurotragi* has been associated with bovine cerebral theileriosis (Tzaneen disease) in southern Africa (de Vos, Bessenger and Banting, 1981). It has also been shown to infect sheep and goats under experimental conditions and may infect a wide range of other artiodactyl wildlife hosts (Stagg *et al.* 1983). The parasite is transmitted by *Rhipicephalus* spp. of ticks including *R. appendiculatus* (Norval, Perry and Young, 1992). The parasite is widely distributed throughout eastern, central and southern Africa, and its distribution is assumed to follow that of its tick vectors.

The major importance of *T. taurotragi* infections of cattle is due to the confusion it causes in the differential diagnosis with *T. parva*. *T. taurotragi* and *T. parva* are transmitted by the

same tick vector, *R. appendiculatus*, in which they are indistinguishable by light microscopy. In the field, ticks are often infected with both species (Morzaria, 1989) and serological cross-reactivity has also been documented between *T. taurotragi* and *T. parva* (Grootenhuis *et al.* 1979). A detailed understanding of the epidemiology of *T. taurotragi* infection is still lacking, mainly due to the lack of appropriate diagnostic tools.

#### **1.7.1.4 *Theileria sergenti*/*T. buffeli*/*T. orientalis* complex (Yakimov and Dekhterev, 1930; Neveu-Lemaire, 1912; Yakimov and Sudachenkov, 1931)**

Species of the *T. sergenti*/*T. buffeli*/*T. orientalis* complex are widespread throughout temperate zones and the subtropics, including SSA (Becerra, Eggen and de Rooy, 1983; Uilenberg *et al.* 1985; Kiltz *et al.* 1986; Ngumi *et al.* 1994; Uilenberg, 1995). They are transmitted by ticks of the genus *Haemaphysalis* and have been described as bovine 'cosmopolitan benign' *Theileria*. Their pathogenicity in cattle can vary from almost non-pathogenic to significantly pathogenic. Acute anaemia and, in severe cases, fatalities have been reported in grazing calves of imported stock in Japan and Korea (Minami *et al.* 1980; Uilenberg, 1981; Sugimoto, 1997). The parasites are associated with low parasitosis of schizonts (Uilenberg, 1981), while elevated piroplasm parasitaemia is of most importance in infection. The morphologies of the bovine 'cosmopolitan benign' *Theileria* are indistinguishable by microscopy. The taxonomy and nomenclature of this group of parasites is controversial and confusing despite numerous studies (Irvin, 1987; Norval, Perry and Young, 1992; Kawazu *et al.* 1992a, 1992b; Fujisaki, *et al.*, 1994; Katzer *et al.* 1998b, 1998c; Kim *et al.* 1998; Kakuda *et al.* 1998).

#### **1.7.1.5 *Theileria annulata* (Dschunkowsky and Luhs, 1904)**

*T. annulata* is a parasite of cattle and water buffalo (*Bubalus bubalis*) where it causes mild disease in water buffalo but severe disease in cattle, especially susceptible taurine breeds. In cattle the disease is known as the Mediterranean or tropical theileriosis and is transmitted by ticks of the genus *Hyalomma* (Uilenberg, 1981). *T. annulata* is distributed throughout the Mediterranean basin, the Middle East and Asia. In Africa, its distribution is confined to North Africa, the Nile river valley and southern Sudan.



#### **1.7.1.6 *Theileria lestoquardi* (Dschunkowsky and Urodschevich, 1924)**

*T. lestoquardi* (syn. *T. hirci*) causes theileriosis in sheep and goats (Uilenberg, 1995). The disease is considered equivalent to that caused by *T. annulata* in cattle. *T. lestoquardi*, like *T. annulata*, is transmitted by the tick *Hyalomma anatolicum anatolicum*, and its distribution is probably similar to that of *T. annulata* (Brown, Hunter and Luckins, 1990). It is of notable importance in Iran, Iraq, Jordan and Syria, where the parasite can be highly pathogenic in susceptible sheep (Brown, Hunter and Luckins, 1990). Piroplasm parasitaemia may be high (Uilenberg, 1981) and both the schizont and piroplasm stages are incriminated in the pathogenesis, and in fatal cases anaemia is almost always present.

#### **1.7.1.7 *Theileria equi* (Mehlhorn and Schein, 1998)**

*T. equi* (syn. *Babesia equi*) is a pathogenic parasite of equids in the tropical and sub-tropical areas of the world. The disease, equine theileriosis, is characterised by apathy, intermittent fever, haemoglobinuria, jaundice, abortions and sometimes death (Brown, Hunter and Luckins, 1990). It is estimated that most of the world equine population are infected with *T. equi* (Schein, 1988). Infected horses can become carriers and international trade regulations require horses to be seronegative for *T. equi*.

#### **1.7.1.8 Other *Theileria* species infecting wildlife**

Most wild ungulates are probably infected with *Theileria* (Neitz, 1957; Irvin *et al.* 1973; Grootenhuis and Young, 1981; Grootenhuis, 1999), but attempts to transmit these parasites to livestock have generally failed, except for *Theileria* parasites from African buffalo, eland and waterbuck (Grootenhuis, 1999). *Theileria* isolated from sable antelope (*T. sp.* Sable) appears to be a novel species (Stoltz and Dunsterville, 1992; Allsopp *et al.* 1994; Chansiri *et al.* 1999). An apparently novel *Theileria* species from African buffalo (*T. sp.* African buffalo) has also been isolated (T. Kakuda, unpublished data). *Theileria* infecting deer (*T. cervi*) have also been identified (Chae *et al.* 1999b).

### **1.7.2 Transmission and life cycle of *Theileria* parasites**

Details of the life cycle are not available for all species of *Theileria*, however *T. parva* life cycle has been described in considerable detail (Norval, Perry and Young, 1992). An account of the *T. parva* life cycle is described here to serve as an example for the genus, with some details of other species. A simplified life cycle of *Theileria* is shown

schematically in two distinct types of hosts, one in the tick vector and the other in the bovine host (Figure 15).

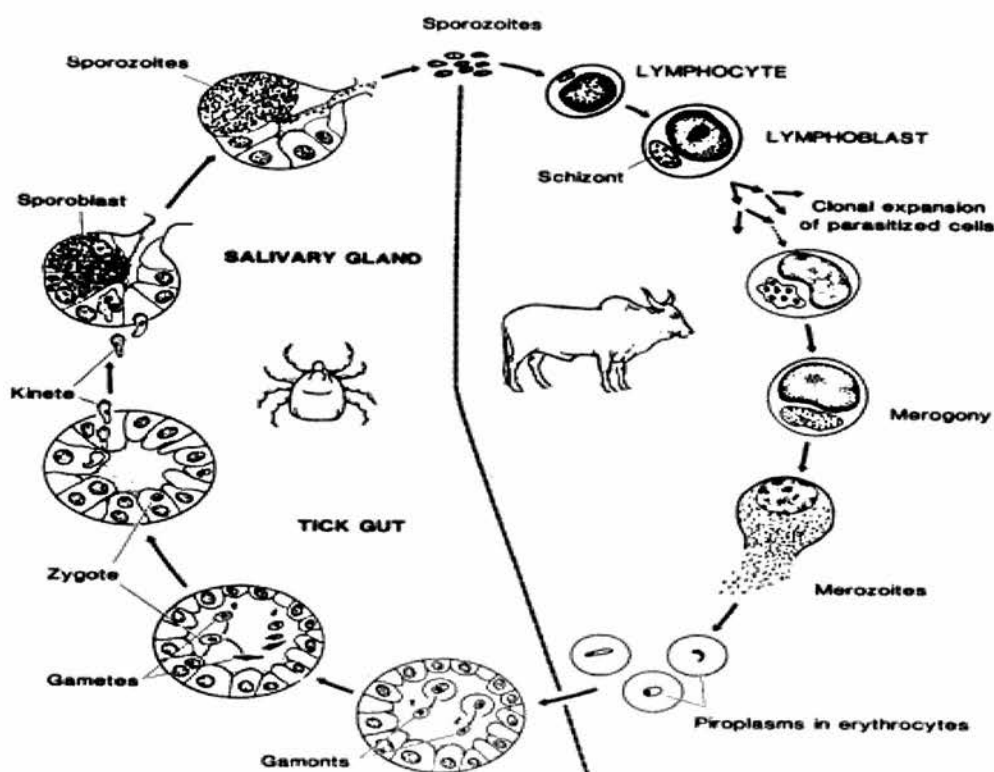


Figure 15: The life cycle of *Theileria* parasites

### 1.7.2.1 Sporozoite invasion of lymphoid cells

Mature sporozoites are small (0.75-1.5µm diameter) tick salivary gland forms of the parasite that are covered by a surface coat. The sporozoites are injected into cattle with the saliva of the tick during a blood meal where they rapidly attach to and establish infection within specific populations of lymphoid cells of the host. *T. parva* sporozoites invade and develop within subsets of T- and B-lymphocytes, suggesting that the invasion is mediated by specific interactions between the sporozoite and the host cell (Baldwin *et al.* 1988; Shaw, 1997). Following binding to the host cell, the sporozoite rapidly enters the cell by ‘zippering’ of the parasite and host membranes (Shaw, 1997). Discharge of material from organelles of the apical complex (also known as the rhoptries) and microspheres then occurs concomitantly with the lysis of the encapsulating host cell membrane and the

appearance of a host-derived microtubule array around the sporozoite (Fawcett, Musoke and Voigt, 1984; Shaw, Tilney and Musoke, 1991).

#### **1.7.2.2 Intra - lymphocytic schizogony**

The newly invaded sporozoite develops into the schizont, which is a multinuclear syncytium, associated with host cell blastogenesis and uncontrolled clonal proliferation of the parasitised cell. Daughter cells retain the infection through synchronous division of the parasite and host cell. The ability of *Theileria* schizonts to transform the infected host cell is known to occur in several species including *T. parva*, *T. annulata*, *T. lestoquardi* and *T. taurotragi*. The molecular basis of this transformation is however not fully understood although it has been shown that parasitised cells display persistent translocation of the active form of transcriptional activator nuclear factor  $\kappa$ B (NF $\kappa$ B) to the nucleus (Ivanov *et al.* 1989). This activator nuclear factor is involved in proliferation and protection against natural cell death (apoptosis) (Dobbelaere *et al.*, 1999). Infected cells also show constitutive expression of interleukin 2 (IL-2) and its receptor in infected cells, resulting in autocrine growth.

It has also been demonstrated that *T. parva*-infected T cells have enhanced activities of tyrosine kinase and casein kinase II (CKII), both of which are involved in signal transduction and may be involved in the transformation of these cells (Eichhorn and Dobbelaere, 1994; Ole-MoiYoi *et al.* 1992, 1993). Lymphoblastogenesis and clonal expansion of infected cells, including concurrent schizont division, occurs at a rapid rate giving rise to a 10-fold increase in infected cells every three days (Irvin, *et al.*, 1982). The proliferation of the infected lymphocytes and subsequent destruction of overgrown lymphoid tissue, which then metastasize into other tissues, gives rise to the main pathogenic effects of ECF (Radley *et al.*, 1974).

#### **1.7.2.3 Merogony**

Twelve to fourteen days after infection the schizonts of *T. parva* begin to undergo merogony, the differentiation to the merozoite stage. This process involves the formation of large numbers of individual, uninucleate merozoites from a multinucleate syncytial schizont (Mehlhorn and Schein, 1984; Shaw and Tilney, 1992). At the end of merogony the host cell contains merozoites and a residual body (Shaw and Tilney, 1992). Differentiation

is complete when the host lymphoid cell and its plasma membrane, breaks down, liberating mature merozoites of 1-2  $\mu\text{m}$  in diameter (Shaw and Tilney, 1992). Shiels and others, (1994) have shown that in *T. annulata* the early part of the differentiation process to the merozoite is reversible, and is followed by a commitment step which is an irreversible phase of differentiation. The commitment to differentiate might be dependant upon the parasite attaining a threshold level of “regulator factors” (Shiels, 1999).

#### **1.7.2.4 Entry and development of merozoites inside the erythrocytes**

The merozoites invade erythrocytes by a mechanism that is similar to sporozoite invasion of lymphocytes, (Shaw and Tilney, 1995). Following invasion, escape of the merozoite from the encapsulating erythrocyte membrane occurs concurrently with discharge of the rhoptries, (Shaw and Tilney, 1995). The piroplasms can divide by schizogony, (Conrad, Denham and Brown, 1986; Fawcett *et al.* 1987; Mehlhorn and Schein, 1998), to produce a maximum of four merozoites. The process is similar to intra-lymphocytic schizogony, (Conrad, Denham and Brown, 1986; Fawcett *et al.* 1987; Shaw and Tilney, 1992). Intra-erythrocytic schizogony is a common feature of many species of *Theileria* (Norval, Perry and Young, 1992). However, in *T. parva* this process is very limited, so the bulk of piroplasms must be related to the numbers of merozoites produced by intra-lymphocytic merogony (Conrad, Denham and Brown, 1986; Fawcett *et al.* 1987).

#### **1.7.2.5 Parasite development within the tick**

Infected erythrocytes ingested by the tick are lysed and the piroplasms are released into the tick gut where a proportion of the piroplasms undergo further development into sexual stages known as micro and macro-gametes (Mehlhorn and Schein, 1984). Ray bodies (believed to be microgamonts) forms (strahlenkörper stages) of the parasite appear soon after repletion of the tick. They have been identified in infections of *T. parva*, *T. annulata*, *T. taurotragi*, *T. mutans* and *T. velifera* (Schein *et al.* 1975; Schein, Warnecke and Kirmse, 1977; Young *et al.* 1980; Warnecke *et al.* 1980; Mehlhorn and Schein, 1984). Ray bodies with up to four nuclei appear which then produce stages thought to be uninucleate gametes from the fifth day after tick feeding.

Following syngamy a spherical zygote develops in the tick gut epithelium to form a motile kinete (Mehlhorn and Schein, 1984). The kinete is liberated from the epithelium into the

haemocoele, from where it invades E cells of Type III salivary gland acini at the time of larval and nymphal moulting of the ticks (Schein, Warnecke and Kirmse, 1977; Fawcett, Buscher and Doxsey, 1982). During the following instar, activated by a rise in body temperature through feeding and ingestion of blood, the parasite develops into a syncytium. This stage, known as the sporoblast, produces up to 50,000 sporozoites in each infected acinar cell through a process similar to merogony (Mehlhorn and Schein, 1984). The life cycle process ends with the emission of the sporozoites into the acinar duct, (Shaw and Young, 1995).

*Theileria* parasites are transmitted trans-stadially, but not trans-ovarially, by the two and three-host ixodid ticks, the 'hard ticks'. *Theileria* infection is acquired by larval or nymphal stages of the ticks during feeding and then transmitted by either the nymphs or adults. While the basic life cycle is probably similar for each species of *Theileria*, there are some clear differences in the relative importance of particular life cycle stages. For example, *T. parva*, *T. annulata*, and *T. lestoquardi* divide extensively as schizonts in lymphoid cells, while *T. mutans* and species of the *T. buffeli/sergenti/orientalis* complex divide extensively as merozoites in erythrocytes. Erythrocytic merogony also occurs in *T. annulata* and *T. lestoquardi*, but is probably very limited in *T. parva*, (Norval, *et al.*, 1992). Lymphoid cells infected with schizonts of *T. parva*, *T. taurotragi*, *T. annulata*, *T. lestoquardi*, *T. sp. Sable* and *T. sp. Buffalo* can be propagated indefinitely *in vitro* since the parasites have the ability to transform the host cells. Schizont-infected lymphoid cells of other *Theileria* species, including *T. mutans* and those of the *T. sergenti/T. buffeli/orientalis* complex, are however refractory to *in vitro* culture. Intra-erythrocytic stages of *Theileria* have not been successfully cultured *in vitro*, except for those of *T. equi* (Holman *et al.* 1994; Zweygarth, *et al.*, 1995).

The complex life cycle of *Theileria* (Figure 15) takes place in an ungulate host and a tick vector and contains both sexual and asexual phases. Schizogony and merogony are phases of asexual division within the ungulate host while gamogony is a sexual phase in the tick vector. However, sporogony which is an asexual reproduction phase occurs inside the tick vector and develops from the zygote to the generation of numerous sporozoites. The differentiation of *Theileria* from one stage to the next is required for transmission to, and establishment within, the host and vector.

### **1.7.3 The pathology of the disease caused by *T. parva* infection in cattle**

Mortality rates following infection may approach 95% due to classical ECF caused by *T. parva* in susceptible taurine cattle (Morzaria *et al.*, 1988). However, in Zebu cattle (*Bos indicus*) raised in endemic areas, mortality may be quite low. The course of the disease also varies according to the parasite strain involved and the size of the sporozoites inoculum (Irvin and Mwamachi, 1983; Cunningham *et al.*, 1974). The most acute forms of the disease are usually seen in exotic breeds introduced into enzootic areas where the typical course of the disease takes about 3 weeks. The initial symptoms of a *T. parva* infection usually appear 5 to 8 days after an infective tick bite during which stage the disease can be diagnosed by lymph node biopsy smears.

The early phase of the disease is usually accompanied by high fever and enlargement of lymph nodes (Irvin and Mwamachi, 1983). The animals display apathy or restlessness, loss of appetite and weight, and in lactating animals, there is a drastic decline in milk production. Subsequently, a lympho-destructive phase associated with massive leucopaenia in fatal cases occurs. In terminal cases, pulmonary oedema, which manifests itself clinically as severe respiratory distress occurs. Rapid lympho-proliferation of parasitized cells followed by their subsequent destruction constitutes the main pathological feature of ECF. Even though large numbers of erythrocytes may become infected with piroplasms, this stage does not appear to be pathogenic (Morrison, Taracha and McKeever, 1995).

### **1.7.4 Diagnosis of East Coast fever (*T. parva*) infection**

#### **1.7.4.1 Clinical diagnosis**

In Africa, the diagnosis of ECF is currently predominantly based on the presence of what are considered to be characteristic clinical signs such as pyrexia, lymphadenopathy, pulmonary oedema, subcutaneous oedema, diarrhoea, lachrymation and corneal opacity (Norval, Perry and Young, 1992). Giemsa staining and microscopic examination of lymph node biopsy and blood smears has been used to detect the presence of *T. parva* in infected animals (Stagg *et al.*, 1981). The test can detect parasites during patent infection, but frequently fails to detect parasites in carrier animals, and is therefore unsuitable for evaluating the role of carrier animals in the epidemiology of theileriosis. Species differentiation is particularly difficult in the case of mixed infections using the Geimsa test



alone because it cannot always differentiate between the piroplasms of other *Theileria* species due to morphological similarities (Norval, Perry and Young, 1992).

#### **1.7.4.2. Immunological diagnosis**

The indirect fluorescent antibody test (IFA), using schizont antigen has been widely used for the detection of serum antibody in infected animals (Goddeeris *et al.*, 1982). This method is not specific as there is significant cross-reactivity with other parasite species, particularly *Theileria taurotragi* (Grootenhuis *et al.*, 1979). An enzyme-linked immunosorbent assay (ELISA) using a recombinant polymorphic immunodominant molecule isolated from *T. parva* (Toye *et al.*, 1991, 1996) has demonstrated sensitivity and specificity of between 94% and 98% using experimental and field sera (Katende *et al.*, 1998).

#### **1.7.4.3. Molecular diagnosis**

DNA probes have proved valuable in distinguishing species and stocks of *Theileria*, which may be difficult or impossible to distinguish morphologically or serologically. The application of polymerase chain reaction (PCR) using primers derived from *T. parva* Tpr repetitive sequences, (Conrad *et al.*, 1987a; Allsopp and Allsopp, 1988), has been successful in detecting parasites of several stocks in the blood of carrier cattle (Bishop *et al.*, 1992). PCR-based assays for detecting cattle persistently infected with *T. parva* are currently favoured over microscopic analysis of Giemsa-stained blood smears and probe-hybridization methods because of superior sensitivity and higher throughput.

### **1.7.5. Control of ECF**

#### **1.7.5.1. Vector control**

Over the years the control of ECF and other tick borne diseases have been based on the frequent application of acaricides either through dipping, sprays or as pour-ons to control the tick vector. However this control strategy has become unsustainable due to a variety of reasons such as the high cost of acaricides, the development of resistance to acaricides by ticks, concerns relating to environmental contamination with toxic residues and the potential risk to human health, (Norval, Perry and Young, 1992). Alternative methods of disease control such as barrier fencing, quarantine of animals, rotational burning of pastures

and planting of tick-killing or repelling plants have been used in the past, with varying results. In the case of smallholder dairy farms, the shift toward intensive management with cattle confined to zero-grazing units, where the only possibility for vector contact is through the introduction of tick-infested fodder, has helped to reduce the incidence of tick-borne diseases in general (Maloo *et al.*, 1994; Gitau, *et al.*, 1997).

An alternative tick control measure is to induce host resistance through vaccination against ticks (reviewed in Morrison, 1989). It is envisaged that effective immunization of livestock against both ticks and tick-transmitted pathogens could significantly reduce the use of acaricides (Norval, Perry and Young, 1992; Opdebeeck, 1994; reviewed in Willasden, 1999). Recombinant mid-gut-associated molecules of *Boophilus microplus* ('concealed antigens' – Bm86 and Bm91), have been shown to give protective immunity against *B. microplus* infestation in cattle (Rand *et al.*, 1989; Tellam *et al.*, 1992; Riding *et al.*, 1994; Willasden *et al.*, 1996). TickGARD™ is a commercial vaccine based on Bm86 marketed in Australia. Currently there are ongoing research efforts to identify protective salivary gland antigens that could be used to produce a second-generation tick vaccine against *R. appendiculatus*.

In Uganda, TBDs have been largely controlled through the application of acaricides in dips or as sprays (Shannon, 1977) aimed at controlling the vectors and protecting the exotic breeds. However, it soon became clear that the untreated indigenous breeds which constituted 98% of the cattle in the country, were acting as reservoirs for the ticks and tick-borne diseases infections for the improved cattle. Consequently, in 1968 the government enacted a law that made tick control mandatory for all breeds of cattle without regards to the economic consequences to the farmers. However, due to civil war between 1971-1985, coupled with socio-economic problems, tick control was seriously disrupted, a situation that resulted in the current drop of 50% of dips in the country while the rest of the economically able farmers have resorted to hand spraying with indiscriminate application of all the available acaricides. This has impacted seriously on the epidemiology of TBDs in the country because of tick resistance to acaricides. Due to inappropriate legislation and lack of partnership between the farmers, extension workers, policy-makers and the scientists dealing with TBD control the current methods have been poorly implemented. The costs of acaricides are also expensive as well as being toxic and harmful to the

environment. The limitations experienced with the current control programmes have led to the development of alternative options that are profitable, technically feasible, socially and environmentally sound as well as being capable to generate benefits to farmers. Through the use of tick models such as CLIMEX and DYMEX a lot of information is being generated on climate matching for different areas in the country, potential distribution of the vectors and the relevant vector control requirements specific to the locality. CLIMEX was used to simulate the potential distribution of the three species of ticks of economic importance in Uganda in various localities as well as to identify localities in the country that have similar climatic conditions to form the basis for planning tick control strategies. DYMEX on the other hand was used to design the most cost effective tick control strategies for *R. appendiculatus* in selected locations

#### **1.7.5.2 Chemotherapeutic control of ECF**

Successful chemotherapy of ECF has been shown to be due to suppression of schizonts during the incubation period with tetracycline (Neitz 1957). Anti-theilerial hydroxynaphthoquinone drugs, *parvaquone* (Clexon, Wellcome, U.K.) and halofuginone lactate (Terit, Hoechst, Germany) (Schein and Voigt, 1979; McHardy, *et al.*, 1985), have chemotherapeutic activity against the pathogenic stages of the parasite in the mammalian host (Dolan *et al.*, 1984, Dolan, 1986a). However for effective treatment of theileriosis, these drugs need to be complemented by early diagnosis since they are not totally effective in the advanced stages of infection. Furthermore, the drugs are expensive and their availability is often erratic. The cost of treating cattle for East Coast fever has been calculated to be US\$60 per animal (Mukhebi *et al.*, 1995).

#### **1.7.5.3 Immunization of cattle against ECF**

Currently, the only practical method of immunization is the infection and treatment method (ITM) (Radley *et al.*, 1975) that involves the inoculation of cattle with a previously characterized and potentially lethal dose of sporozoites of *T. parva* and simultaneous treatment with an antibiotic (usually a long-acting formulation of oxytetracycline). This technique is based on the observation that cattle recovering from the infection are immune to any subsequent re-challenge (Neitz, 1957; reviewed by Irvin *et al.*, 1981). Immunity, which results from recovery, appears to be long lasting (Burridge *et al.*, 1972), and is

thought to be dependent on generation of CD8<sup>+</sup> MHC class I restricted cytotoxic T cells that lyse schizont-infected cells (McKeever *et al.*, 1994; reviewed by Morrison, Taracha and McKeever, 1995). However when cattle are challenged with heterologous parasites immunity may be incomplete, hence a cocktail of strains is used (reviewed by Irvin, 1987; also in Uilenberg, 1999).

There are however drawbacks to this method of immunization, which limit its use. The requirement for a cold chain for delivery of cryopreserved sporozoites to the field in combination with poor infrastructure and the additional requirement for veterinary expertise, coupled with the high cost of preparing and preserving sporozoites are major limitations to the sustainable deployment of ITM. In addition, not all stocks are equally amenable to control by tetracycline. Not all strains of *T. parva* cross-protect, and therefore a cocktail of parasites derived from strains that in combination provide cross-protection must frequently be used (Morzaria *et al.*, 2000).

Vaccinated animals typically remain long-term carriers of *Theileria* parasites that are infective to ticks with the potential to create novel strains of *T. parva* through sexual recombination (Morzaria *et al.*, 1992) with endemic strains. Due to the drawbacks of this live vaccine however, efforts have continued in the development of new sub-unit vaccines based on recombinant antigens of *T. parva*, identified through studies on mechanisms of protective bovine immunological response (Morrison, Taracha and McKeever, 1995; Musoke *et al.*, 1996). A candidate of such a vaccine antigen named p67, which is the major surface antigen found on *T. parva* sporozoites has been identified and its gene cloned and characterized (Iams *et al.*, 1990a; Nene *et al.*, 1992; Nene *et al.*, 1996). A subunit vaccine based on a recombinant version of this gene has undergone both field and laboratory trials and has been shown to provide approximately 70% protection against challenge by both homologous and heterologous cattle-derived *T. parva* stocks in the laboratory (Musoke *et al.*, 1992; Nene *et al.*, 1996). Although the mechanism of immunity induced by p67 is unclear, antibodies to p67 can neutralise sporozoite infectivity for bovine lymphocytes *in vitro* (Dobbelaere, Shapiro and Webster 1985; Musoke *et al.*, 1984, 1992; Nene *et al.*, 1992, 1996). It is envisaged that p67 will form a major component of a multivalent sub-unit vaccine against ECF in combination with other antigens, particularly those that induce class I-restricted CTL responses specific for schizont-infected lymphocytes.

## 1.8 OTHER TICK-BORNE PATHOGENS OF CATTLE

The other tick-borne pathogens of cattle in sub-Saharan Africa include: *Anaplasma* species causing anaplasmosis, *Ehlichia* species causing tropical bovine Ehlichiosis (Nofel or Nopel) and *Babesia* species causing babesiosis.

### 1.8.1 Anaplasmosis

Anaplasmosis is a group of virulent and infectious diseases caused by several species of rickettsial parasites, *Anaplasma* (Figure 16 a) that affect both wild and domestic ungulates. The parasites are usually transmitted by ticks but may also be transmitted mechanically by biting flies such as *Stomoxys* and *Tabanids*. The general pathological manifestations of the disease include progressive anaemia, which may be acute or mild that ends in cachexia and death. Infected calves undergo mild infection with little or no mortality but the yearlings do have a more severe disease and eventually recover. However, the adults succumb to the disease with 50% mortality, resulting in severe losses in endemic areas. The animals start with depression, indolence and fever which can go up to 40-41°C. Milk production normally drops rapidly in lactating animals, as the weight loss follows progressively. Recovered animals normally remain carriers for life. The parasites are exclusively intra-erythrocytic where they are surrounded by a vacuole invaginated from the host cell. The infection starts with an 'initial body' which grows to become an elementary body. The elementary body multiplies by binary fission to produce more initial bodies that accumulate in the vacuole until they leave the host cell to parasitize other host cells.

Anaplasmosis tends to occur following infection with theileriosis or babesiosis and appears as a prolonged convalescence. Demonstration of the organisms in Giemsa stained blood smears is confirmation of infection. Sero-diagnosis has also been used (Knowles, *et al.*, 1996) as well as DNA probes that are used to determine infection and prevalence in cattle. Acute anaplasmosis is often treated with oxytetracycline at 5-10mg/kg body weight. The disease is often controlled by the methods that target the vectors. However, immunization of animals by inoculation with one virulent and attenuated species against the other in endemic areas can also be done in young animals but could be disastrous with adults.



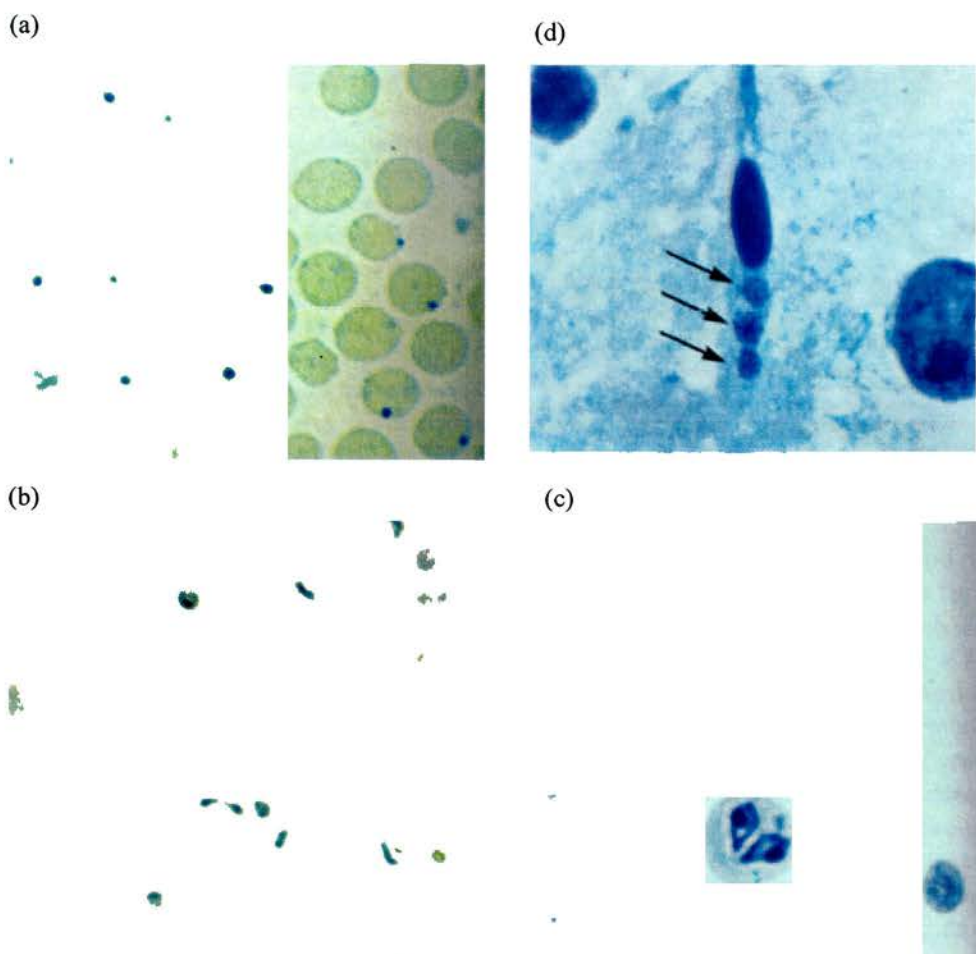


Figure 16: Other tick-borne parasites of cattle, (a) *Anaplasma marginale*, (b) *Babesia divergens*, (c) *B. major*, in the bovine red blood cells and (d) *Cowdria ruminantium*, in the bovine capillary vessel (Johannes Kaufmann, 1996).

Different species of *Anaplasma* parasites are morphologically indistinguishable and can only be differentiated by their location within the host cells as either marginally or centrally located in the cell. *A. marginale* is responsible for the malignant anaplasmosis of cattle, also known as the gall sickness and is distributed throughout the tropics where its vector hosts are *Boophilus decoloratus* and *B. microplus*. About 80-90% of the parasites can be found in the peripheral blood system. The prevalence of anaplasmosis based on parasitological studies in Africa show 33% in Uganda, 37% in Kenya and Nigeria while sero-prevalence in Uganda has been shown to be 62% (Ssnyonga, *et al.*, 1991; Anon, 1996; Mulei and Rege, 1989; Latif, *et al.*, 1996; Egbe-Nwiyi, 1997).



### 1.8.2 Babesiosis

Babesiosis is a group of diseases caused by *Babesia bovis*, *B. bigemina*, *B. divergens* and *B. major*. *Babesia* species (Figure 16 (b) and (c)) are transmitted by a variety of ticks. *B. bovis* is transmitted by the *Boophilus* species of ticks which are also the vectors for *B. bigemina* while *B. divergens* is transmitted by *Ixodes ricinus* and *B. major* is transmitted by *Haemaphysalis punctata*. In cattle, infections with *B. bigemina*, *B. major* and *B. divergens* cause haemolytic syndrome which includes continuous fever, high parasitaemia, anaemia, icterus, haemoglobinuria and general depression. In dairy cows abortion and agalactia are the early symptoms. *B. bovis* infections result in shock syndrome, high temperature leading to death. Babesiosis is widespread throughout the tropics where it causes heavy losses in exotic non-resistant breeds. Control of the disease includes the use of chemotherapy and immunization with attenuated strains of parasites, as well as vector control.

### 1.8.3 Cowdriosis

Cowdriosis, also known as heartwater, is an infectious, non-contagious disease of ruminants, characterized by high fever, hydropericardium and nervous syndrome. The disease is caused by the parasite *Cowdria ruminantium* (originally known as *Rickettsia ruminantium*, Figure 16 (d)), and is transmitted by *Amblyomma* ticks. Since the vectors of this parasite *A. variegatum*, *A. gemma* and *A. lepidum*, are abundant throughout Uganda, it is always presumed that the disease is endemic in most parts of the country, (Mutugi, *et al.*, 1995). However, the importance of this disease is often underestimated due to the presence of other tick-transmitted diseases such as theileriosis, anaplasmosis and babesiosis, in Uganda, (Uilenberg, 1983). Indeed the first case of natural infection of a goat with this disease in Uganda was only reported recently, (Saimo, *et al.*, 2001) and there is clearly a need to carry out more epidemiological studies countrywide and develop diagnostic tools for field diagnosis.

## 1.9. THE EPIDEMIOLOGY OF TBD IN UGANDA

The distribution of ticks was studied in Uganda in the 1960s and has never been revised since then. With the exception of north – east Uganda where *R. appendiculatus* and *A. variegatum* occur only marginally, the important ticks and TBD are widely distributed all over the country. Ticks and TBDs cause serious debility, morbidity, mortality and production losses in susceptible taurine cattle, their crosses and the indigenous breeds

reared in ECF-free parts of the country but moved into endemic areas. However, the economic losses caused by ticks and TBDs have not been quantified for the different breeds of cattle and livestock production systems in Uganda.

In Kenya, for example, there is a considerable body of recent evidence that the prevalence of *T. parva* infections and reported ECF morbidity, mortality and case-fatality can vary significantly by agro-ecological zone (AEZ), and grazing system (Deem *et al.*, 1993; Gitau *et al.*, 2000). These differences have implications for both the impact and control of ECF. A conceptual framework for assessing production systems for ECF based on their 'endemic stability' has been developed by Norval *et al.*, (1992) and Perry and Young, (1995) and may be extrapolated to cover Uganda as well.

In endemically stable systems, there is normally an equilibrium between *Theileria* parasite populations and their hosts so that ECF impact through clinical disease is minimal despite a continuous high tick challenge. This is due to acquired immunity against a potentially lethal challenge with local *T. parva* stocks. In endemically unstable situations, ECF impact can be considerable, both through direct loss from ECF, and in costs associated with control. In situations with a sufficient level and continuity of challenge by infected ticks, one option for disease control is to move toward or maintain endemic stability.

### **1.9.1 The carrier state of *Theileria parva***

In order to understand the epidemiology of ECF and the disease it causes as well as assessing the impact of immunization with live parasite stocks, an objective assessment of the carrier state of *T. parva* in the mammalian host is paramount. It is also important to make a distinction between persistent infection (pre-munition), in the field, which occurs due to a continuous challenge from infected ticks, sterile immunity in which parasites are cleared by the host-immune system and the tick-transmissible carrier state, particularly in cattle. Sterile immunity refers to cattle that have recovered from a *T. parva* infection and are solidly immune to a homologous challenge. However, such animals can sometimes succumb to heterologous challenge, owing to antigenic polymorphism in *T. parva* (in Morrison and McKeever, 1998).

The carrier state of *T. parva* refers to a state in which cattle have survived a primary infection and still maintain parasite stages such as the schizonts or piroplasms within their peripheral circulation. Carrier animals that maintain the infectious parasite piroplasms in circulating blood at levels that are high enough are capable of infecting ticks, which may in turn transmit the parasites to susceptible hosts, (Young *et al.*, 1986; Maritim *et al.*, 1990). Such carriers are important for maintaining *T. parva* infection in the field because the tick vector, *R. appendiculatus*, cannot transmit the parasite transovarially. Not only is the carrier state important for maintenance of infection, but it may also be necessary for maintenance of immunity.

An accurate assessment of the prevalence of carrier animals could therefore be useful in determining the immune status in naturally infected and vaccinated cattle, and hence determine the requirement for maintenance of immunity. It is possible for a *Theileria*-infected host to develop a primary parasitosis and parasitaemia, which are subsequently cleared by the host, leaving it without any parasites but immune (sterile immunity) (reviewed in Boulter and Hall, 1999). However in many *T. parva* infected animals, infection persists after recovery. This is probably maintained by limited multiplication of schizont-infected lymphocytes, some of which undergo merogony to produce merozoites, transforming into piroplasms, which are then infective to the erythrocytes (Maritim *et al.*, 1989; Kariuki, 1991). The presence or absence of circulating schizonts (schizont carrier) or piroplasms that are tick-transmissible, determines whether an animal is a carrier at a given time.

Live vaccination against ECF, by infection with a sporozoite stabilate and simultaneous treatment with a long acting tetracycline, also induces a carrier state with most *T. parva* stocks (Maritim *et al.*, 1989; Bishop *et al.*, 1992; Kariuki *et al.*, 1995). The carrier state in cattle induced by immunization requires detailed investigation, to determine whether it may result in the establishment of “foreign” strains in a particular area and whether the carrier state is required for the maintenance of immunity against *T. parva* infection. In addition to induction by vaccination cattle can also develop a carrier state after treatment of ECF by chemotherapy (Dolan *et al.*, 1984; Dolan, 1986b), including therapy with buparvaquone (Butalex<sup>®</sup>).

The different types of carrier- state induced following infection with *T. parva* have previously been described, (Norval, Perry and Young, 1992) but the proportion of field challenged and recovered animals that are persistently infected with *T. parva*, and the effectiveness with which they transmit infection to ticks over time is currently unknown since carrier animals are not possible to detect by classical diagnostic methods. However, several PCR assays for the detection of *T. parva* in carrier cattle have been developed and shown from previous studies to be capable of detecting *T. parva* DNA in experimentally-infected carrier animals and in cattle in field situations (Bishop *et al.*, 1992; Watt, *et al.*, 1998; Skilton *et al.*, 2002). The assay, based on primers derived from the p104 antigen, has proved most sensitive (Skilton *et al.*, 2002). PCR has been used to detect the presence of low-level infections in cattle and ticks, in the context of *T. annulata* infections (d'Oliveira *et al.*, 1995, 1997; Ilhan *et al.*, 1998; Roy *et al.*, 2000), and also carrier states induced by other tick borne pathogens (Gale *et al.*, 1996).

### **1.9.2 Integrated control of ticks and tick-borne diseases (ICTTBD) in Uganda**

Ticks and tick-borne diseases are the single most important impediment to the development of the cattle industry in Uganda. Prior to the introduction of the susceptible taurine breeds into the country in the 20<sup>th</sup> century there was a solid endemic stability to TBDs in the indigenous cattle. Tick burdens in cattle were traditionally controlled by hand picking or grass burning but the problem of TBDs was largely unknown to local people until the introduction of high yield susceptible breeds when these diseases, especially ECF, caused heavy mortalities. The need for massive tick control was however only realized after one of the species of ticks – *R. appendiculatus* - was implicated as being the vector of ECF in 1911. Since then, 10 genera and 72 species of ixodid ticks have been recorded in Uganda, (Matthysse and Colbo, 1987).

The concept of ICTTBD was developed with the specific objective to minimize disease occurrence by controlling tick vector numbers and TBD in an economical and an environmentally sustainable manner. It utilizes a combination of complementary methods that are sustainable and economically viable, rather than a single method such as the intense use of acaricides, (de Castro, 1997). Available methods which can be combined for integrated control include strategic use of acaricides at economic thresholds, biological control of ticks, quarantine and control of livestock movement, as well as the use of hosts



resistant to ticks and TBD, immunization against TBD, chemotherapy and epidemiological manipulation, (Norval, *et al.*, 1992). A prerequisite for successful implementation of ICTTB is an improved understanding of the epidemiology of TBD through the use of improved molecular and immunological tools for parasite characterization and assessment of prevalence.

## 1.10 THE STUDY AREA

### 1.10.1 Uganda

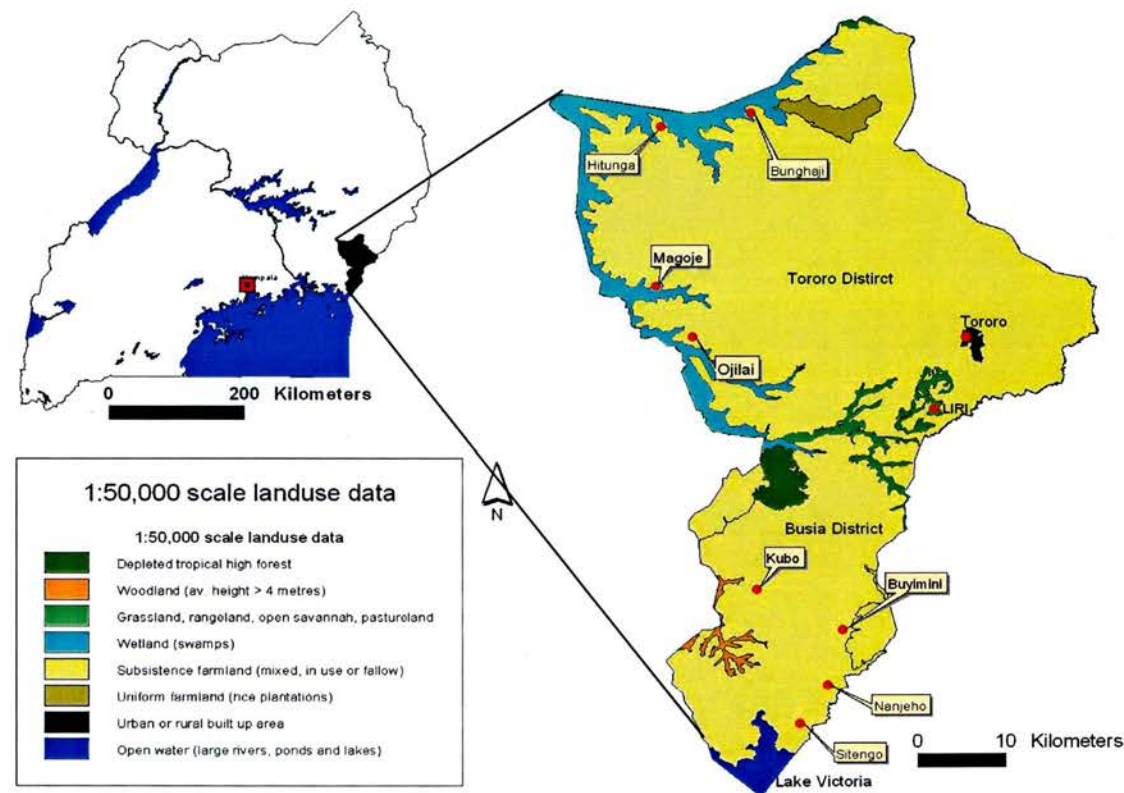


Figure 17: Map of Uganda and the study area villages in Tororo and Busia

Uganda is a landlocked country, bordered by Sudan to the North, DRC to the west, Rwanda and Tanzania to the South, and Kenya to the East, Figure 17. The country lies astride the equator between latitudes 4° .0' North and 1° .30' South of the equator, and longitudes 30° .0' East and 35° .0' East of Greenwich, covering an area of 242,554 km<sup>2</sup>. The topography of much of it can be classified as a plateau, with numerous small hills and valleys and extensive savannah plains. The entire country lies above 900m above sea level, generally

sloping from South to North. The country lies in a cradle of mountains on its east border with Kenya, Mt. Elgon, Mt. Moroto to the North East, and to the South-Western, the Rwenzori Ranges rising to an altitude over 2000m. The country is well watered with close to 17% or 51,000 km<sup>2</sup> of its area dedicated to swamp or open water. Much of the country lies in the 'Inter-lacustrine Region' (between the lakes) of Africa that receives abundant rainfall, and is rich in tillable land, a major determining factor in settlement of the area.

Vegetation in Uganda is extremely diverse, a result of the different micro-climates of the country. Vegetation zones can be roughly classified according to the rainfall zones and are generally; the Lake region, the Northern Region, and the Highlands of the South-East defined according to the climate of the particular areas. The country's natural environment provides good grazing for cattle, sheep, and goats, with indigenous breeds dominating most livestock. Smallholder farmers own about 95 percent of all cattle, although several hundred modern commercial ranches have been established during the 1960s and early 1970s in areas that had been cleared of tsetse-fly infestation. Ranching was successful in the late 1960s, but during the upheaval of the 1970s many ranches were looted, and most farmers sold off their animals at low prices to minimize their losses. In the 1980s, the government provided substantial aid to farmers, and by 1983 eighty ranches had been restocked with cattle, a factor that has since been established to be responsible for the resurgence of sleeping sickness in SE Uganda (Fevré, *et al.*, 2001).

Cattle rustling, especially along the Kenyan border, also depleted herds in some areas of the northeast. The government hoped to increase the cattle population to 10 million by the year 2000. To do this, it arranged a purchase of cattle from Tanzania in 1988 and implemented a US\$10.5 million project supported by the Kuwait government to rehabilitate the cattle industry. The government also approved an EEC-funded program of artificial insemination, and the Department of Veterinary Services and Animal Industry tried to save existing cattle stock by containing diseases such as bovine pleuro-pneumonia, hoof-and-mouth disease, rinderpest, and trypanosomiasis.



### **1.10.2. Tororo District**

Formerly known as Bukedi, Tororo district is one of the districts that already existed at independence. It borders Pallisa district in the north, Mbale in the north east, Iganga in the west, Busia and L. Victoria in the south, and Kenya in the east. The district covers an area of approximately 2,600 sq. Km with an altitude of 1,097 to 1,219m above sea level. The district has moderate rainfall but high average temperatures (Figure 18). It has a population of approximately 600,000 people. The main economic activity in the district is subsistence farming with cotton as the main cash crop. There is also cross border trade with Kenya. The health services include a 226 bed capacity Tororo hospital and seven other health centres. Livestock population in Tororo includes 159,000 cattle, 24,000 pigs, 15,000 sheep and 88,000 goats, (Rwabwoogo, 2002).

### **1.10.3 Busia District**

Busia district was created in 1997 from Tororo district. It borders Kenya to the east, Tororo district to the north and Iganga district to the west and continues into L. Victoria. The area of the district is 733 sq. Km and a population of approximately 200,000 people mostly living in rural areas. The population is unevenly distributed with areas near the south and along the border with Kenya having a higher density than the areas bordering Tororo in the north and Bugiri in the west. The district lies in the Lake Victoria shore region with relatively high and uniform relief rainfall at the shores and on the islands but declines as one moves inland.

Like Tororo, the main economic activity in Busia district is subsistence agriculture with some cash crops such as ginger, cotton and sunflower being grown. Fishing is a major industry in the district as well as the cross-border trade between Kenya and Uganda. There are nine health units supporting the population, including health centres, dispensaries, maternity centres, sub-dispensaries and aid posts. The district has animal population of approximately 28,819 cattle, 9767 pigs, 5,318 sheep and 22,700 goats, (Rwabwoogo, 2002). The district has similar rainfall and temperature pattern as Tororo district.

### 1.11 THE OBJECTIVES OF THE STUDY

In sub-Saharan Africa the major diseases of livestock are transmitted by vectors. The most important of these diseases include the tsetse-transmitted trypanosomiasis caused by *T. congolense*, *T. brucei* and *T. vivax* and the tick-transmitted theileriosis, babesiosis, anaplasmosis and cowdriosis. These vector transmitted pathogens lead to a combined loss in revenue to both the small holder farmers and governments of more than \$4 billion annually. Control of these diseases is expensive and complex, hence the need to understand their transmission and dynamics which in turn require better understanding of their epidemiology in order to rationalize economical control strategies.

The overall objective of this study was to understand the epidemiology of the vector-transmitted diseases in cattle reared under mixed farming management system in the villages of SE Uganda. In order to achieve this, two of the most commonly used technologies, microscopy and molecular, for pathogen detection and species characterization were evaluated for their sensitivity and specificity and utilized in detecting the parasites in the blood of cattle. Despite its low sensitivity and lack of specificity in detecting disease pathogens, microscopy was used due to its wide applicability and low cost that is affordable by most disease surveillance teams working in the field. Molecular methods were used due to their high sensitivity and specificity in relation to microscopy, despite their higher cost.

Specifically the study aim was to make use of the available molecular markers and methodologies to characterize the two main groups of vector-borne parasites – tick and tsetse transmitted parasites circulating in cattle in Busia and Tororo Districts of south east Uganda and to quantify the impact of trypanosome prevalence in the area. The study also sought to assess the impact of drug intervention on the prevalence of trypanosomiasis in indigenous cattle based on molecular screening method of trypanosome detection in cattle. This would ultimately provide information that could lead to better management of disease control strategies.

## CHAPTER TWO

### 2 THE STUDY DESIGN

The study areas covering the eight villages from Tororo and Busia districts of SE Uganda, are shown in Figures 19 and 20. The study areas are known to be endemic for vector transmitted animal pathogens, (Lancien, *et al.*, 1990; Okoth, *et al.*, 1991) that lead to massive economic losses to the farmers.

The two districts of Tororo and Busia where the study was conducted are known to be predominantly infested with *Glossina fuscipes fuscipes* and, to a limited extent, by *G. pallidipes*, (Lancien, *et al.*, 1990; Okoth, *et al.*, 1991; Magona, *et al.*, 1997 and Okuna, *et al.*, 1999), which transmit both human and animal trypanosomiasis. The main tick species found in this area include *Rhipicephalus appendiculatus*, *R. evertsi evertsi*, *Boophilus decoloratus* and *Amblyoma variagatum*, (Okello-Onen, *et al.*, 1999) which transmit *Theileria* parasites, *Anaplasma*, *Babesia* and *Cowdria* to livestock.

Agricultural production system in the study areas is mainly by smallholder farmers who produce food and cash crops integrated with livestock keeping, (Okello-Onen, *et al.*, 2003). Cattle which include the Zebu and Sanga breeds (Table 2) are kept under traditional communal grazing management, either free grazing on communal pastures or tethered during the day and tied at home or in 'bomas' at night. Calves below six months age are always tethered around the homestead

In traditional management systems the majority of cattle owners do not normally undertake any organized disease control measures such as cattle dipping or regular treatment of trypanosome infected animals. This is largely due to economic constraints and the prohibitive costs of drugs. It was therefore expected that the impact of the diseases to be studied would be high enough to justify the study.

2.2 THE CLIMATE OF THE STUDY AREAS

Busia district lies in the Lake Victoria shore region with relatively high and uniform relief rainfall at the shores and on the islands which declines as one moves inland, while Tororo district has moderate rainfall but high average temperatures. The rainfall pattern follows a bimodal annual type ranging between 1200-1500mm distributed in March to May (long rains) and September to November (short rains) interspersed with dry seasons (December to February and June to August) Figure 18. The daily mean maximum temperature is 27°C while the daily mean minimum temperature is 15°C, although small seasonal variations in rainfall and temperatures occur between districts, (Ford and Katondo, 1976).

The climatic conditions together with the presence of swamps and marshland in the study areas are also favourable for the survival of snails which are intermediate hosts for *Fasciola gigantica*, (Magona *et al.*, 1999) as well as the continual survival of other helminth species in pastures.

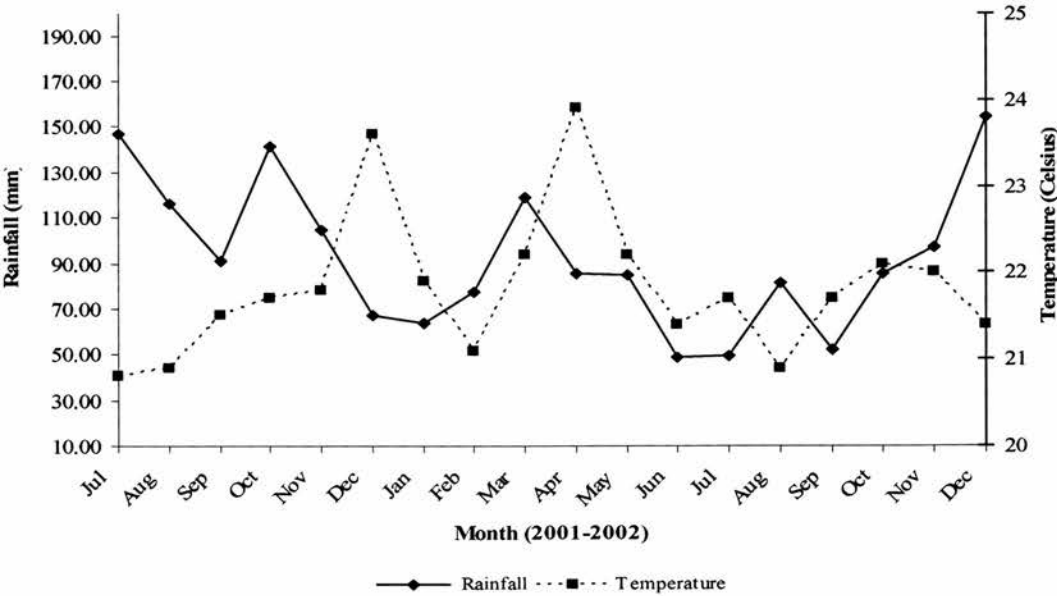


Figure 18: Rainfall and temperature patterns in the SE Uganda region during the study period, (Uganda Meteorological Department, Tororo, 2002)

## 2.3 SELECTION OF THE STUDY VILLAGES AND ANIMALS

Selection of the study villages was 'purposive', since the areas are known to have tsetse and trypanosomiasis, (Lancien, *et al.*, 1990; Okuna, *et al.*, 1996). Individual village characteristics were also considered during the selection. The availability of more than 80 cattle from each village based on the cattle census conducted at the time was also considered. An equally important consideration was that each village had to be sufficiently isolated from the others with no overlap during cattle grazing (Figures 19 and 20).

### 2.3.1 Village characteristics

Tororo villages selected for the study were Bunghaji, Hitunga, Magoje and Ojilai. The locations and relief features of these villages are shown on Figure 19. The type of farming in these villages is mainly subsistence mixed with cattle rearing. Cattle are mainly grazed on communal areas which spread into the swamps or open grassland. The villages were located approximately 10 km or more away from each other.

- Bunghaji village lies to the north of the district and is in close proximity to a swamp. The vegetation is mainly Savannah grassland with *Lantana camara* shrubbery but no forest cover.
- Hitunga village lies to the north-west of the district and is located at the edge of a swamp, has open Savannah grassland and with minimum forest cover.
- Magoje village lies to the western part of the district and is also in close proximity to a swamp with papyrus type of vegetation. The village is also in close proximity to woodland vegetation, with trees approximately 4 metres in height, as well as shrubbery, mainly *L. camara*.
- Ojilai village lies to the south-western part of the district and is also in close proximity to a swamp with open Savannah grassland and tropical high forest to the north of the village.



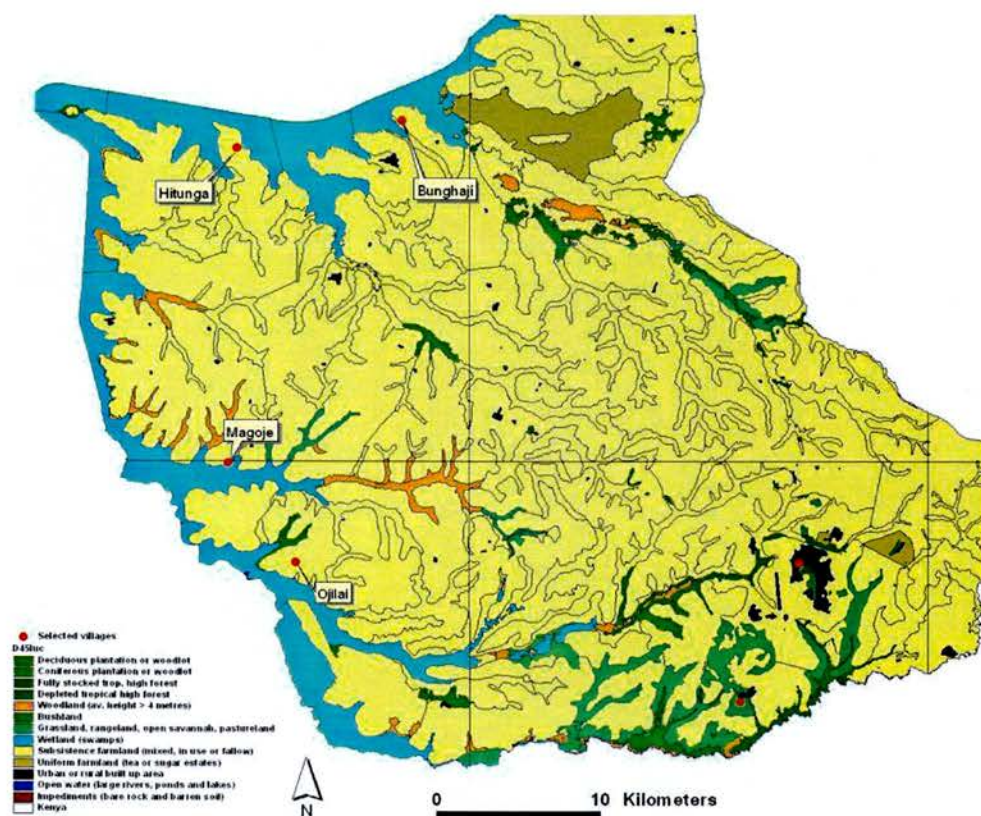


Figure 19: Map of Tororo district showing the locations and the relief features of the four study villages.

The villages of Busia district selected for the study were Buyimini, Kubo, Nanjeho and Sitengo and their locations and relief features are shown in Figure 20.

- Buyimini village lies to the east of the district but is situated a little further away from the nearest swamp compared to the other villages. It is however within the subsistence farmland and is surrounded by forest cover.
- Kubo village lies to the mid-western part of the district, at the forest edge within the mixed farmland. The village is also in close proximity to open Savannah grassland and a swamp.
- Nanjeho village lies to the south-east of the district in close proximity to a swamp. It is situated mainly in the subsistence farmland and is also in close proximity to a forest cover.

- Sitengo village lies to the south of the district, surrounded by woodland vegetation and is also in close proximity to a swamp.

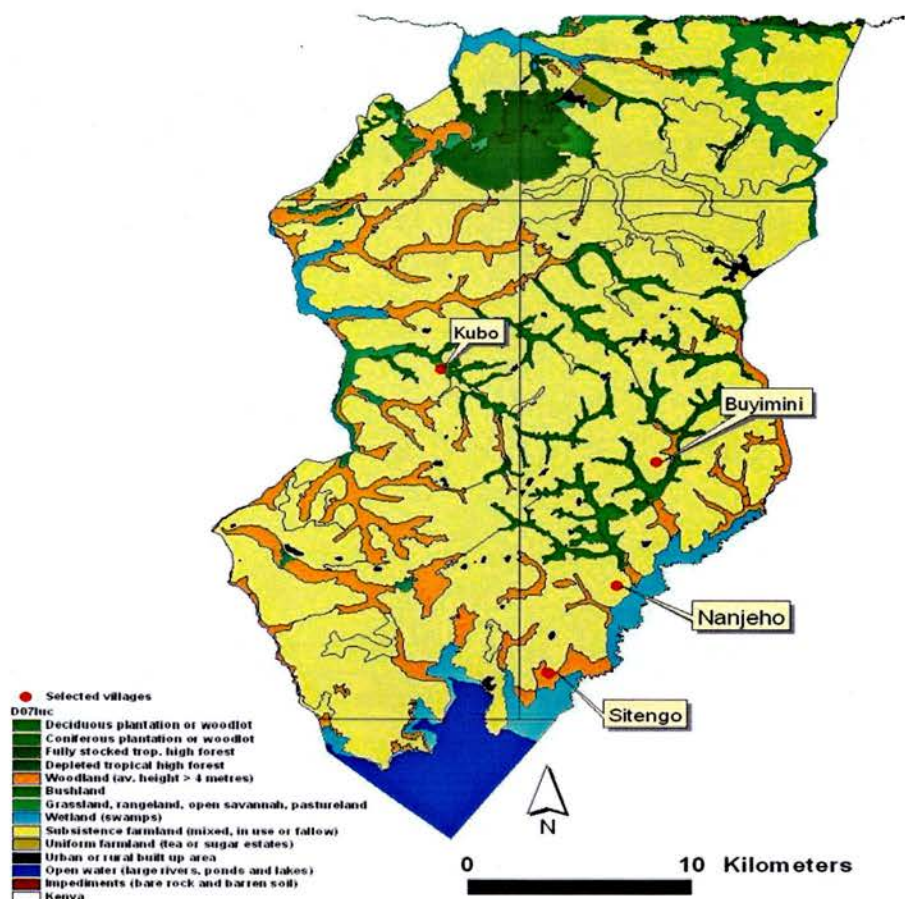


Figure 20: Map of Busia district showing the locations and relief features of the four study villages.

### 2.3.2 Selection of cattle

Cattle census, including owner information, herd size and age of the animals, was conducted with the help of the owners and local village administrators to guide the recruitment of animals into the intervention study. The initial village data were recorded in data sheets as shown in the example for Buyimini East and West, in Table 5. The objectives of the studies were explained to the cattle owners, following which their permission to use the animals for the study was sought and received. Individual cattle were selected following the ‘systematic sampling method’ (Thrusfield, 1995), according to the order in which the

owners presented them at the designated collection centres in each village. A total of 80 Zebu cattle of variable ages and both sexes, whose owners were willing to participate in the study, were randomly selected from each village herd and ear-tagged with assigned specific numbers. Information such as estimated age or date of birth, cattle owner, herd size and sex were recorded as shown in Table 5.

Table 5: Design of the initial data sheet for farmer recruitment into the study. (M – Males, F – Females)

Buyimini East												
Farmer No	Farmer Name	Herd Size	< 1 year		1 - 2 years			> 2 years			Total Tagged	
			M	F	M	F	M	F				
1	Wanyama Malikiswa	7	1	1	1			3			6	
2	Egondi Zemolina	3			1	1		1				
3	Buluma Pasikali	5	1	1	1						19	
4	Tambiti Jackson	23	4	3	4			1		9		
5	Tanga Yokana	1						1				
6	Wamalma Stephen	5		2	2			1				
7	Wanyama Fred Osinya	1	1								(25)	
8	Mangoli Ojiambo	2			1		1					
(47)												
Buyimini West												
Farmer No	Farmer Name	Herd Size	< 1 year		1 - 2 years			> 2 years			Total Tagged	
			M	F	M	F	M	F				
9	Wafula Ngolobe	5	1		2						9	
10	Onyango James	11		1	8							
11	Oguttu K	1								1	5	
12	Mangini Hillary	1					1					
13	Oguttu Ngolobe	4	2	2								
14	Owori Ngolobi	3	1	2								
15	Wanyama Olumbe	1	1								(14)	
16	Okello Vincent	2	1	1								
17	Amborosi Musungu	6	1	2						3		
18	Taaka Ansilini	1		1							5	
19	Wabwire Mukaga	4	2									
20	Aswangi Helen	2		1						1		
21	Kitari Vincent	1		1							(14)	
(42)												



## **2.4 BASELINE STUDY**

Baseline study conducted in the eight villages of Tororo and Busia districts (Figures 20 and 21) was designed to determine the diagnostic capabilities of microscopy and molecular techniques in detecting vector transmitted parasites. These techniques were to be used in the subsequent drug intervention studies. The study was also designed to provide the baseline prevalence and proportions of trypanosome species and tick transmitted parasites, the most common endemic diseases of cattle under mixed farming production system. The data collected during the baseline study covered a wider range of parasites and parameters including helminthes, clinical examination and body condition score as reported in Magona's PhD thesis (Glasgow University, 2004). Clinical data were collected by Dr. Ian Anderson while microscopic screening was done by the staff at LIRI Parasitology unit. However, the main focus of this study was on prevalence of vector transmitted diseases, namely, the tsetse – transmitted trypanosomes and the tick – transmitted parasites, mainly due to their economic importance.

## **2.5 DRUG INTERVENTION STUDIES**

The drug intervention studies were designed to run over a period of 14 months in Busia and 16 months in Tororo villages. Although the selection of ear-tagged cattle took into consideration the representation of all herds in each village and the owners' consent to participate in the study, it had to be age-weighted to compensate for the possible higher rates of drop out in younger animals. The same animals selected and ear-tagged during the baseline study in the villages of the two districts of Tororo and Busia were followed up in the subsequent sampling and data collection. Similar field data was collected during the subsequent visits as the ones collected at the baseline. All calves born to ear-tagged animals were recruited into the study.

## **2.6 THE STUDY DESIGN**

Seven days prior to the treatment for the intervention studies sample collection was done after which the animals were ear-tagged and randomly assigned to treatment groups as is listed below. The treatments were replicated in two villages of each district as follows:

- Mass treatment with 1mg/Kg body weight, Isometamidium chloride (ISMM)) only Magoje in Tororo and Nanjeho in Busia, targeting trypanosomes.



- Mass treatment with 10mg/Kg body weight, long-acting Oxytetracycline only in Bunghanji in Tororo and Sitengo in Busia, targeting theileriosis.
- Mass treatment with both Isometamidium chloride and Oxytetracycline as above in Hitunga in Tororo and Kubo in Busia.
- Controls – no prophylactic treatments - Ojilai in Tororo and Buyimini in Busia.

The control villages were included to provide continuous data from naturally infected animals without drug intervention. The data was then used as the existing disease prevalence status from which the impact of treatment intervention could be assessed.

### **2.6.1. Veterinary support**

During the intermediate visits to the villages by the veterinarian any animals, including those that were not part of the study, which showed clinical signs of infection with trypanosomes, theileriosis or worm infection, were treated. The animals which showed signs of trypanosomiasis were treated with 3.5mg/kg body weight diminazene aceturate (Berenil<sup>®</sup>, Hoechst, Germany). Treatment of trypanosomiasis was also based on the level of haemoglobin concentration against a minimum threshold of 8g/dl which is an indication of anaemia (Johns and Lewis, 1989). The animals which showed signs of theileriosis as determined from clinical examination were treated with 10mg/kg long acting Oxytetracyclin while those which showed signs of helminths infection were drenched with ivermectin. Each treatment given to any animal during the visits was also documented in the field record sheets.

### **2.6.2 Sampling visits**

Village visits were made by the field team from Livestock Research Institute (LRI), Tororo, led by a veterinarian once every four weeks. Contact persons who had preferably been working as local administrators were nominated by the communities participating in the study to help mobilize the cattle owners and to ensure smooth running of the study. During the visits, ear-tagged animals were either sampled in the owners' homestead or at the designated collection centres. The sampling timetable was given to the contact persons as well as prior notice in advance of the sampling date in each village.

Following treatment, sampling was carried out in all villages once every four weeks, on day 21, 49, 77, 105, 133, 161, 189, 217, 245, 273, 301, 329, 357, 385, 413 and 441 in Tororo and 21, 49, 77, 105, 133, 161, 189, 217, 245, 273, 301, 329, 357 and 385 in Busia, as shown in Table 7. However, follow up surveys were conducted in the middle of each sampling month to monitor the animals' health through clinical examination and assessment of the body condition score, (Nicholson and Butterworth, 1986) and carry out treatment on sick animals as part of the free veterinary services provided to the participating farmers. In Tororo villages the study was initiated in the month of July 2001, while in Busia the study started three months later, in the month of October the same year.

## **CHAPTER THREE**

### **3. MATERIALS AND METHODS**

#### **3.1 FIELD BASED METHODS**

##### **3.1.1 Clinical examination**

During the monthly visits full clinical history of each animal as well as the general herd condition such as abortions or stillbirths or deaths was obtained from the owners. The ages were determined by asking the owners the date of birth or how old they knew the animals were. General physical examination was done on all cattle in the study by the veterinarian and cross-checked with the owners' descriptions. Parotid, prescapular and prefemoral lymph nodes were palpated to assess whether they were enlarged. The skin coat was examined for any signs of roughness or starring as well as for the presence of ecto-parasites such as the lice and ticks. The tick species, if found, were identified and recorded. Oral, vulvular and conjunctival mucous membranes were examined for pallor or petechial haemorrhages. Anaemia was assessed by measuring the haemoglobin using the battery operated hand held Hemocue (HemoCue AB, Angelholm, Sweden).

The animals were examined for signs of diarrhea and ocular or nasal discharge. Rectal temperatures were taken using a digital thermometer to determine signs of pyrexia. Body condition score was performed based on the method described by Nicholson and Butterworth (1986), to assess the extent of emaciation. The scoring system followed assessed the condition as F – fat, M – medium and L – lean. Each of the categories was further graded as F+, F and F-; M and M-; L+, L and L-. F+ was represented by a score of 9 while L- was scored as 1. Body condition scoring is a measure of the extent to which fat is stored or the muscle mass has declined and involves examining anatomical parts such as the tail head, brisket, hump, transverse processes of the lumbar vertebrae, hips and ribs, the depth of the sub-lumbar fossa as well as the muscle mass between the tuber coxae and tubar ischii. All observations related to clinical examination and serology were recorded in the field data collection sheets for each village, Table 6 and reported in Magona's thesis, (Glasgow University, 2004).

Table 6: Field data collection sheet used in all the villages.

No	Tag No	H/cue	Wt	C. S	MM	LN	AC	SL	Diarr.	Disch	K. L	H (O)	Hh (V)	Lice	T (Rh)	T (Amb)	T (Boo)	RT	Diag	Treat
1																				
2																				
3																				

Key: H/cue - Hemocue, Wt - weight C.S. - condition score, MM - mucus membrane, LN - lymph node, AC - animal coat, SL - skin lesion, Diarr - diarrhea, Disch - discharge, K.L. - ketone level, H(O) - health (owner), H(V) - health (vet), T(Rh) - ticks (*Rhipicephalus*), T(Amb) - ticks (*Amblyoma*), T(Boo) - ticks (*Boophilus*), RT- rectal temperature, Diag - diagnosis, Treat - treatment.

### **3.1.1 Measurement of the haemoglobin concentration**

The measurement of haemoglobin (Hb) was achieved by collecting 10 µl of blood from needle-pricked ear vein onto the cavity of the microcuvettes that were inserted into the measuring chamber of the HemoCue. The HemoCue is a haemoglobinometer system that measures haemoglobin at two wavelengths, 565 and 880 nm, as methaemoglobin without dilution. The sodium azide and sodium nitrate, deposited onto the microcuvettes as dry reagents, lyse the red blood cells releasing haemoglobin. The sodium nitrate reduces the haemoglobin iron from the ferrous to the ferric form which then combines with the azide to form the measurable methaemoglobin, (von Schrenk, *et al.*, 1986). The concentration of haemoglobin was read from the digital readout of the machine and recorded in the field data sheets.

### **3.1.2 Collection of faecal, lymph and blood samples**

Faecal materials were collected from the rectum of each animal in the study, placed in labeled plastic bags and dispatched on ice to the laboratory for further processing. Lymph node biopsies were taken from the parotid lymph nodes with 5 ml syringes and G16 needles and smears made from animals that showed signs of pyrexia, enlarged lymph nodes or had other signs of *T. parva* infection. The smears were labeled with the animal number and date of sampling, air dried and taken to the laboratory for further processing and examination microscopic examination for tick-borne parasites.

Ear-tagged animals were bled from the jugular vein into two pre-labeled vacutainers, one with heparin and the other one without, using gauge 20 multisampling venoject needles (Becton-Dickinson Vacutainer system, UK). Twenty millilitres of blood was collected from each of the animals, 10 mls in heparinized vacutainer tubes and the other 10 mls in plain vacutainer tubes. The heparinized blood was used for the preparation of buffy coat samples on FTA filter cards for further screening of parasites by PCR amplification. The blood was also used for microscopic examination of parasites by the buffy-coat phase contrast method (Murray, *et al.*, 1977), and for estimation of the packed red cell volume, (PCV), following centrifugation in micro haematocrit capillary tubes. The blood in plain vacutainers was used for the preparation of serum samples used in serological screening of tick borne diseases (Magona, 2004, thesis, Glasgow University). Ear-vein blood was also used to prepare both thick and thin smears that were subsequently used in trypanosome species



identification as well as for examination of tick-borne parasites by microscopy. The number of animals sampled monthly from each village is shown in Table 7. Sampling was normally done on designated days between 8.00 am and 12.00.

Table 7: Number of animals sampled monthly from each village in Tororo and Busia districts in 2001-2002. (NS – not sampled)

Tororo		Sampling months and days															
Month	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov
Village	-7	21	49	77	105	133	161	189	217	245	273	301	329	357	385	413	441
Bunghaji	79	NS	48	66	60	56	52	73	71	64	75	72	51	71	78	70	72
Hitunga	82	NS	78	80	80	76	76	80	79	75	76	76	67	72	79	75	55
Magoje	79	NS	72	73	73	66	60	65	57	55	60	70	64	63	72	62	55
Ojilal	79	NS	48	66	60	56	52	73	71	64	75	72	57	71	78	70	72
Sub-Total	319	NS	246	285	273	254	240	291	278	258	286	290	239	277	307	277	254
		4380															
Busia																	
Month	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	May	Jun	Jul	Aug	Sep	Oct	Nov		
Village	-7	21	49	77	105	133	161	189	217	245	273	301	329	357	3851	Total	
Buyimini	80	77	75	66	58	74	66	77	68	72	68	72	70	60	61	1044	
Kubo	80	75	80	75	65	71	67	56	74	55	58	61	69	70	65	1021	
Nanjeho	79	74	79	65	66	66	71	59	64	76	66	69	67	66	65	1032	
Sitengo	80	78	81	81	80	77	78	79	80	79	78	77	79	76	73	1176	
Total	319	304	315	287	269	288	282	271	286	282	270	279	285	272	264	4273	

## **3.2 LABORATORY BASED METHODS**

### **3.2.1 Microscopy**

Microscopic examination method was used because it is quick and relatively inexpensive and allows for the organisms to be visualized as well as allowing for estimation of the density of parasites. The method was used to examine faecal samples for worm eggs, stained lymph node biopsies for *T. parva* macroschizonts and both stained thick and thin blood smears for trypanosomes and tick borne parasites (*Anaplasma*, *Babesia* and *Theileria*). It was also used for examination of buffy coat preparations for trypanosomes.

#### **3.2.1.1 Examination of faecal samples**

Faecal samples from the field were fixed in 1% formalin v/v to prevent hatching of nematode eggs and stored at 4°C until they were examined for worm eggs. The samples were examined under the microscope for strongyle eggs using the McMaster method as described by Hansen and Perry (1994). The samples were also examined for *Fasciola* and *Schistosoma* eggs and the results recorded in the laboratory data sheets. The results of these observations are reported by Magona (PhD Thesis, 2004, Glasgow University).

#### **3.2.1.2 Examination of lymph node biopsies**

The lymph node biopsy smears were fixed in methanol for 3 – 5 minutes. The smears were air dried and then stained in 10% Giemsa stain diluted in phosphate buffer pH 7.2 for 30 minutes. The slides were then differentiated with tap water, air dried and covered with DPX mountant with coverslip. The biopsies were examined under the microscope with immersion oil at 1000 magnification for *Theileria parva*. Fifty microscopic fields were examined for *T. parva* macroschizonts in the lymphocytes, considered confirmatory for East Coast fever (Urquhart, *et al.*, 1996).

#### **3.2.1.3 Examination of blood samples**

Heparinized blood samples were used for examination of trypanosomes and tick-borne parasites. Microscopic examination of blood samples for trypanosomes was done by the haematocrit centrifugation technique, HCT (Woo, 1971) and buffy-coat phase contrast methods (Murray, *et al*, 1979; Paris *et al.*, 1982). Plain capillary tubes were filled with 70µl of heparinized blood, sealed at one end and centrifuged at 12,000 RPM in a microhaematocrit centrifuge for 5 minutes. The capillaries were first used to estimate

packed red cell volume (PCV), on a haematocrit reader, (Hawksley, UK). The buffy coat area of the span capillary tubes, (the interface between the packed red cells and the plasma), was examined under the microscope for trypanosomes (Woo, 1971). The capillaries were subsequently cut with a diamond glass cutter at the interface between the packed red cells and the buffy coat and their contents from the plasma side were transferred onto clean glass slides. The preparations were covered with 22x22 mm glass coverslips and at least 50 microscopic fields were examined further for the presence of trypanosomes under the microscope at 250X magnification (Murray, *et al.*, 1979, Paris *et al.*, 1982).

Dried thin blood smears were fixed with methanol for 5 minutes, dried and together with the thick blood smears they were stained with 10% Giemsa stain in phosphate buffer pH 7.2 and differentiated with tap water as with the lymph node biopsy smears. The smears were air dried and examined for *Theileria parva*, *Anaplasma*, *Babesia* as well as for trypanosome species identification under the microscope at 1000 times magnification with immersion oil. The intensity of parasitaemia for theilerial and babesial piroplasms as well as *Anaplasma* was assessed by using a scoring system in which a + represented one organism found in 10 fields while +++ represented one or more organism(s) found per microscope field.

### **3.2.2 Preparation of parasite stabilates**

Whole anti-coagulated blood in heparin as well as buffy coat preparations were also stored in liquid nitrogen with 10% glycerol v/v, in phosphate buffered saline with 10% glucose, (PSG), as stabilate, (Lumsden, 1969). The blood or buffy coat preparations in 1 ml aliquots were mixed with glycerol in labeled cyro-tubes, suspended at the vapour phase over night and then lowered into the liquid nitrogen. The frozen stabilates were transported to the International Livestock Research Institute (ILRI), Nairobi for storage, to be used for future stocks of trypanosome reference materials. Some of the stored frozen blood sample preparations were also used in purifying parasite DNA for screening of *Trypanosoma brucei rhodesiense* and tick- borne parasites by molecular techniques.

### 3.2.3 Molecular based techniques

Although many studies use microscopy for screening of animals for parasitic infections this method lacks sensitivity in detecting low parasitaemia in cattle, a common characteristic in naturally infected animals. The technique also lacks the specificity required to characterize various parasite species, sub-species and strains infecting cattle, thus it cannot distinguish pathogenic from non-pathogenic species of parasites. Molecular based techniques such as polymerase chain reaction (PCR) amplification and reverse line blot (RLB) assay were used for screening of trypanosomes and tick-transmitted parasites in the buffy coat and blood. The techniques allow for characterization of individual species of parasites with unique genomic DNA sequences. The techniques are also much more sensitive than traditional microscopy, since many of the markers target DNA molecules that occur in multiple repeats and copy numbers in the genome of an organism. Trypanosomes were screened from cattle buffy coat samples on FTA filter cards, (Whatmann Biosciences). However, *T.b. rhodesiense* and the tick-borne parasites were screened from purified genomic DNA preparations from frozen blood.

#### 3.2.3.1 Preparation of samples for trypanosome screening by PCR amplification

Buffy-coat samples were prepared by centrifugation of the remaining heparinized blood samples in a Beckman centrifuge (USA), with swing out rotors at 3,000 RPM. Buffy coat preparations were collected from the interface between the packed red cells and the plasma. Using Pasteur pipettes, 200µl of the buffy coat was collected and spread onto pre-labeled FTA filter cards. Each card allowed four samples to be spread on the four circles. The cards were air dried at room temperature for at least one hour before they were sealed in envelopes and shipped to the UK for analysis.

The FTA filter cards were processed for PCR amplification of trypanosome species, according to the manufacturer's protocol with some modifications. Eight 1.2 mm punches were made, for each animal, with a Harris punch tool on a cutting mat and collected into 1.5 ml Eppendorf tubes. Eight punches were also collected from blank clean cards to serve as negative controls in each set of PCR amplification. The samples together with the blanks were washed 3 times with 1.4 ml FTA purification reagent and then 3 times with 1x Tris-EDTA buffer, each wash being mixed on a roller mixer for 5 minutes. Washed



punches`were placed in individually labeled PCR reaction tubes with lids open and dried at room temperature for 1 hour. The samples were then ready for incorporation of PCR master mixes prepared according to each amplification protocol for each trypanosome species and type - specific primers.

### **3.2.3.2 Preparation of samples for *T.b. rhodesiense* and *Theileria parva* p104 screening by PCR amplification**

Genomic DNA for the screening of *T.b. rhodesiense* and for p104 PCR was purified from stored frozen blood samples. The DNA was extracted from heparinized frozen cattle blood according to the QIAamp® DNA Blood Mini kit, (QIAGEN, Germany). The blood was thawed at room temperature and 200µl was mixed with 20µl of QIAGEN protease in 1.5 ml eppendorf micro-tubes and mixed by vortexing. To the mixture, 200µl of buffer AL from the kit was added and vortexed and then incubated at 56°C for 10 minutes before adding 200µl of 96-100% ethanol and the mixture vortexed. The mixtures were loaded onto the spin columns (provided in the kit) and centrifuged at 6,000 g (800rpm) for 1 minute in an eppendorf mini-centrifuge. The DNA bound to the resin in the spin columns was washed with 500µl of buffer AW1 from the kit and centrifuged at 6,000g for 1 minute. The DNA was further washed with 500µl of buffer AW2 from the kit and centrifuged at 20,000g (14,000rpm) for 3 minutes. DNA elution was done by adding 100µl of buffer AE from the kit to the columns and centrifuged at 6,000g for 1 minute into 1.5 ml collection tubes.

### **3.2.3.3 Preparation of DNA samples for screening of tick-borne parasites by reverse line blot (RLB) assay**

Heparinized frozen whole blood samples from cattle in the villages of Tororo district were used for screening tick-borne parasites by the reverse line blot assay. Frozen blood was thawed at room temperature and 200µl aliquots drawn from each sample was placed into a 1.5 ml Eppendorf tube. The samples were washed three times with phosphate buffered saline (PBS) pH 7.4 (137 mM NaCl, 0.27 mM KCl, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.18 mM KH<sub>2</sub>PO<sub>4</sub>), by centrifugation at maximum speed in an eppendorf centrifuge for 5 minutes. The washed cells were re-suspended in 100µl of PCR mixture (10mM Tris-HCl [pH8.0], 50mM KCl, 0.5% Tween 20, 100µg/ml of proteinase-K added just before use) and incubated overnight

at 72°C. The samples were then heated for 10 minutes at 100°C to inactivate the proteinase K and centrifuged for 2 minutes, then stored frozen at -20°C until required.

### 3.2.3.4 PCR amplification for trypanosomes

Screening of samples for various trypanosome species, *T. brucei*, *T. congolense* and *T. vivax* in cattle was done with species and type specific primers listed in Table 9. Since the successful amplification of nucleotide sequences by PCR is highly empirical as any given pair of oligonucleotide primers, some optimal set of conditions such as the polymerase enzyme, primer as well as the salt (Mg<sup>2+</sup> ) concentration and the temperature cycle profile had to be established.

However, the PCR cocktails were prepared by adding 1mM dNTP final concetration, the required amount of primer sets for each trypanosome, 0.04U/µl genomic *Taq* polymerase (Sigma) in 1 times PCR buffer, (50mM KCL, 10mM Tris-HCl (pH 9.0), 0.1 Triton® X-100) with 1.5mM MgCl<sub>2</sub>) all in a volume of 25µl, (Picozzi, *et al.*, 2002). A set of purified genomic DNA from *T. brucei*, *T. vivax*, *T. congolense* Savannah type, *T. congolense* Kilifi type, *T. congolense* Forest type and *T. congolense* Tsavo type, Table 8, were included to serve as positive controls every time the screening was done. The PCR amplifications were done on the DNA Dyad Engine (MJ Research), thermal cycler. The amplicons were electrophoressed and viewed under uv following staining with ethidium bromide. All the control DNA samples were generously provided by Dr. Phelix Majiwa, formerly of ILRI, Nairobi.

Table 8: Purified trypanosome DNA used as positive controls

<i>T. b. brucei</i>	ILTat 1.2 (50ng/µl stock, used at 1pg per reaction)
<i>T. vivax</i>	ILDat 1.2 (50ng/µl stock, used at 1pg per reaction)
<i>T. congolense</i> Savannah type	IL1180 (50ng/µl stock, used at 5 pg per reaction)
<i>T. congolense</i> Forest type	IL3900 (50ng/µl stock, used at 5 pg per reaction)
<i>T. congolense</i> Tsavo type	* (50ng/µl stock, used at 5 pg per reaction)

\*Gene cloned into pBlueScrip (Majiwa, *et al.*, 1993)

*T.b. brucei* IL16E1 (ILTat 1.2) was isolated from bovine blood from Uhembo village in, Kenya, in 1964, (Onyango, *et al.*, 1966) and has undergone several passages in mice, (Nantulya, *et al.*, 1984). *T.vivax* IL305 (ILDat 1.2) was isolated from a cow in Zaria, Nigeria, in 1973, (Leefflang, *et al* 1976) and has also undergone several passages and adaptation in mice and rats, (Barry and Gathuo 1984). *T. congolense* Savannah type (IL1180) was isolated from a lion in Serengeti National Park, Tanzania, in 1971, (Geigy and Kaufmann, 1973), and has also been passaged several times in mice, (Nantulya, *et al* 1982). *T. congolense* Forest type (IL3900) was isolated from a dog in Dindenesso, Burkina Fasso, in 1980 and has also been passaged in mice and goats, (Majiwa *et al*, 1993). *T. congolense* Kilifi type (K45.1) was isolated from a cow in Kilifi district, Kenya, in 1982, (Masake, *et al.*, 1987), and has been passaged twice in mice, (Masake, *et al.*, 1988). *T. congolense* Tsavo type was isolated from the proboscis of a female tsetse fly, *Glossina pallidipes*, caught in 1991 near Kichwa Tembo, Ngulia Rhino Sanctuary in Tsavo West National Park, Kenya, (Mihok, *et al.*, 1992). The morphological characteristics and developmental sites in the tsetse fly of this parasite, was found to be indistinguishable from *T. congolense* or *T. simiae* species. However, it did not cause any fatal infection in pigs, a characteristic of *T. simiae* therefore it was classified as *T. congolense* and the isolate designated as GPAL/KE/91/CP 1091, and adapted for growth in culture, (Majiwa, *et al.*, 1993). The detailed history of this trypanosome type was not available since the material was lost in storage, thus the DNA fragment used as control had been cloned in a pBlueScript vector.

Table 9: List of primers used in PCR screening of trypanosomes in cattle. Primers were synthesized by Sigma Genosynth, (UK).

Primer designation	Primer Sequence	Product size/Target	Reference
TBR-1	5'-CGA ATG AAT AA CAA TGC GCA GT-3' 5'-AGA ACC ATT TAT TAG CTT TGT GC-3'	Target a 177-base pair repeat satellite DNA	Sloof, <i>et al.</i> , 1983; Moser, <i>et al.</i> , 1989
TBR-2			
INGI-1(IL0341)	5'-GAT CCG CAG CCG GGC CTG A-3'	Target a <i>T. brucei</i> repetitive element <i>ingi</i> , designed from the middle of the sequence of the <i>ingi</i> element to amplify a band approximately 590 base pairs	Kimmel, <i>et al.</i> , 1987
INGI-2(a)	5'-GGG CCA AGG ACA GTT CCT TGT GG-3'		
TCF (F)	5' - GGA CAC GCC AGA AGG TAC TT -3'	Targets repetitive sequences of <i>T. congolense</i> forest type resulting in a product of 350 base pairs	Gashumba, <i>et al.</i> , 1988
TCF (R)	5' -GTT CTC GCA CCA AAT CCA AC -3'		
TCS (F) IL0344	5'-CGA GAA CGG CAC TTT GCG A-3'	Targets repetitive sequences of <i>T. congolense</i> Savannah type resulting in a product of 316 base pairs	Masiga, <i>et al.</i> , 1992
TCS (R) IL0345	5'-GGA CAA ACA AAT CCC GCA CA-3'		
TCT (F) IL0893	5'-GTC CTG CCA CCG AGT ATG C-3'	Targets a highly repetitive, tandemly arranged satellite DNA sequence from <i>T. congolense</i> Tsavo type, 400bp	Majiwa, <i>et al.</i> , 1993
TCT (R) IL0892	5'-CGA GCA TGC AGG ATG GCC G-3'		
TCK (F) IL0963	5'-GCG GCA GGT CGA CGG ATC-3'	Targets a repetitive DNA specific to kilifi type of <i>T. congolense</i> , 294bp	Masiga, <i>et al.</i> , 1992
TCK (R) IL0968	5'-CCC TCG AGA ACG AGC A-3'		
TWJ (F) IL01264	5'-TCG CTA CCA CAG TCG CAA TCG TCG TCT CAA GG-3'	Tandemly reiterated sequence coding for a Tv27 diagnostic antigen, 400bp	Masake, <i>et al.</i> , 1997
TWJ (R) IL01265	5'-CAG CTC GGC GAA GGC CAC TTG CTG GGG TG-3'		
TVW-1	5'-CTG AGT GCT CCA TGT GCC AC-3'	Targets a 150 base pair fragment specific to <i>T. vivax</i>	Masiga, <i>et al.</i> , 1992
TVW-2	5'-CCA CCA GAA CAC CAA CCT GA-3'		
SRA-1	5'-GAA GAG CCC GTC AAG AAG GTT TG-3'	Targeting <i>SRA</i> - gene 670bp	Welburn, <i>et al.</i> , 2001; Fevré, <i>et al.</i> , 2005.
SRA-2	5'-TTT TGA GCC TTC CAC AAG CTT GGG-3'		

➤ **Screening of cattle for *T. brucei* by PCR amplification**

Cattle were screened for *Trypanosoma brucei* sl. infections with two sets of species-specific primers. The TBR-1 and TBR-2 primers, (Moser, *et al.*, 1989), are specific for the *brucei* species and target a 177 base pair repeat satellite DNA sequence with no apparent coding function, (Sloof, *et al.*, 1983), thus the sequence is not conserved between different trypanosome species. It has been estimated that there may be up to 1000 copies of this satellite DNA per genome of *T. brucei*, (Sloof, *et al.*, 1983). The sequences of the primers are listed in Table 10. The DNA was amplified with 0.2μM forward and reverse primers as follows: step 1 at 94°C for 3 minutes to pre-heat the lid of the thermocycler, step 2 at 94°C for 30 seconds to denature the DNA template; step 3 at 60°C for 30 seconds to anneal the primer to the template; step 4 at 72°C for 30 seconds extension. The amplification was cycled 30 times from step 2 and the final extension was carried out at 72°C for 5 minutes. The samples showing the expected PCR amplification product with TBR primers resulting in the 177bp repeat, Figure 21 (a) lane 1, were regarded as *T. brucei* positives.

The INGI set of primers, which target a *T. brucei* repetitive DNA element *ingi*, (Kimmel, *et al.*, 1987), were used due to their lower sensitivity when compared with the TBR primers since they target a molecule with approximately 200 copies compared to 1000 copies targeted by the TBR primers. The purpose of using these primers was to pick up the animals that could have had high parasitaemia that would have caused PCR amplification inhibition due to excess template. The primers listed in Table 9, were designed from the middle of the sequence of the *ingi* element and amplified a band approximately 590 base pairs. The PCR cocktail was prepared as with TBR primers. The DNA was amplified as follows: step 1 at 94°C for 3 minutes, step 2 at 94°C for 1 minute to denature the DNA template; step 3 at 60°C for 1 minute; step 4 at 72°C for 1 minute for primer extension. The amplification was cycled 30 times from step 2 and the final extension was done at 72°C for 5 minutes. The amplification product resulting in the 590bp *ingi* element, following electrophoresis, is shown in Figure 21 (b) lane 1.

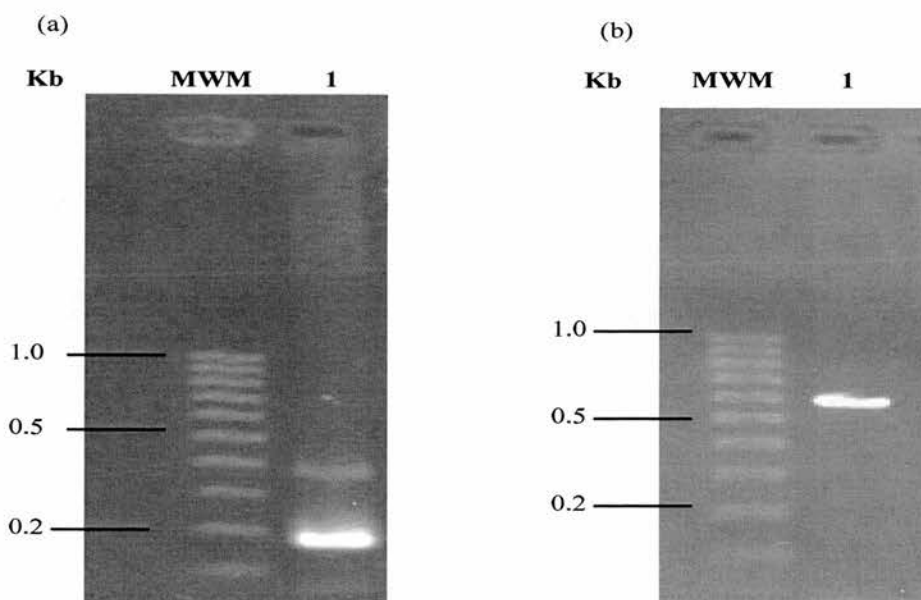


Figure 21: *T. brucei* amplified with, (a) lane 1 - TBR primers targeting the 177bp repeat and (b) lane 1 – INGI primers targeting the 590bp *ingi* element molecule

### ➤ SRA PCR

PCR amplification of the SRA gene forms a basis for identification of *T.b. rhodesiense*, since it has been shown that it does not exist in the other sub-species of *T. brucei* s.l., *T.b. brucei* or *T.b. gambiense*, (Gibson, *et al.*, 2002, McLean *et al.*, 2004). The PCR primers used in this study, as listed in Table 9, were designed to amplify across the deletion and thus distinguish the *SRA* gene from the VSG, resulting in a product of approximately 670 bp.

Five PCR amplifications were performed to detect *T. brucei rhodesiense*, on each sample that was found positive for *T. brucei* by the TBR primers. The *SRA*- gene targeting primers were specific to *T. brucei rhodesiense* which is a single copy gene. The PCR was performed in a volume of 25µl per reaction, including PCR buffer (Qiagen, Crawley, UK) containing a combination of KCl and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and a final concentration of 2.5 mM MgCl<sub>2</sub>, 200µM of each of the 4 dNTPs, 0.2µM of each of the *SRA* primers, (Table 10) and 1.5u of HotStar*Taq*<sup>®</sup> DNA polymerase (Qiagen) were used per reaction. HotStar*Taq*<sup>®</sup>



requires an initial step of 15min at 94°C for activation of the enzyme, prior to starting of PCR cycling. Each reaction was then cycled 50 times at 94°C for 30s, 63°C for 90s and 72°C for 70s and ended by a 10min step at 72°C to ensure complete elongation of all products. The samples that had two bands, the VSG gene band above 1 kbp and the expected band of approximately 0.67 kbp amplification products shown in Figure 22 lane 1 were regarded as positive for *T.b. rhodesiense SRA* gene while the samples that had only the larger than 1 kbp band were regarded as *T.b. brucei*.

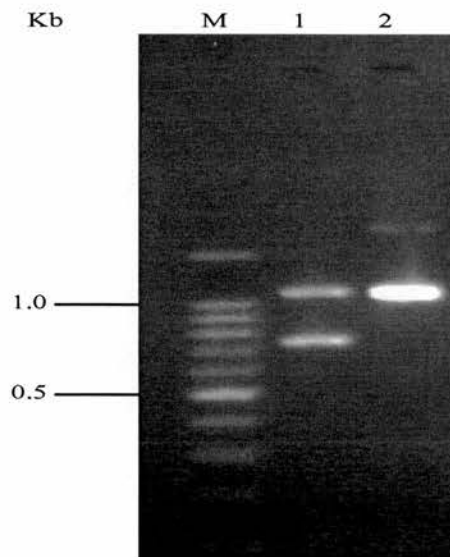


Figure 22: Genomic DNA from *T. b. rhodesiense*, lane 1 (670bp) and *T.b. brucei*, lane 2 (1150bp), amplified with *SRA* –specific primers.

### ➤ **Screening of cattle for *T. vivax* by PCR amplification**

Cattle were screened for *T. vivax* infections with two sets of species-specific primers. The TVW-1 and TVW-2 primers target genomic repeated DNA elements in *T. vivax* and yields a 150 base pair fragment (Masiga, *et al.*, 1992). These primers though highly sensitive and specific, are rather discriminatory, since they have been shown to amplify *T. vivax* from some regions but failed to detect any trypanosomes in other places where *T. vivax* known to exist (Morlais, *et al.*, 2001).The PCR cocktail was prepared with 0.2μM of both forward

and reverse primers (Table 9),, with genomic *Taq* polymerase (Sigma). The DNA was amplified as follows: step 1 at 94°C 3 minutes, step 2 at 94°C 30 seconds; step 3 at 60°C 30 seconds; step 4 at 72°C 30 seconds. Amplification was cycled 30 times from step 2 and the final extension was done at 72°C 5 minutes. The amplified gene product is 150bp as shown in Figure 23 lane 1.

The TWJ-1 and TWJ-2 primers target a tandemly reiterated sequence that encodes for a *T. vivax* diagnostic antigen. This antigen has been shown to be recognized by a monoclonal antibody Tv27 that has been used in antigen detection ELISA (Masake, *et al.*, 1997). The gene contains a micro-satellite motif that has considerable polymorphism within *T. vivax* populations (Morlais, *et al.*, 2001). Although not as sensitive as the TVW, these primers have been shown to amplify *T. vivax* isolates from diverse areas of Africa and South America (Masake, *et al.*, 1997) and from both the vector mouth parts (Lehane, *et al.*, 2000) and the animal host (Morlais, *et al.*, 2001). The sequences of the primers are shown in Table 9. The PCR cocktail was prepared with 0.4μM of both forward and reverse primers and genomic *Taq* polymerase (Sigma). The DNA was amplified as follows: step 1 at 94°C for 3 minutes, step 2 at 94°C for 1 minute; step 3 at 55°C for 2 minutes; step 4 at 72°C for 2 minutes. The amplification was cycled 30 times from step 2 and the final extension was done at 72°C for 5 minutes. The samples which had the expected amplified PCR product of 400bp as shown in Figure 23 lane 2 were regarded as *T. vivax* positives.

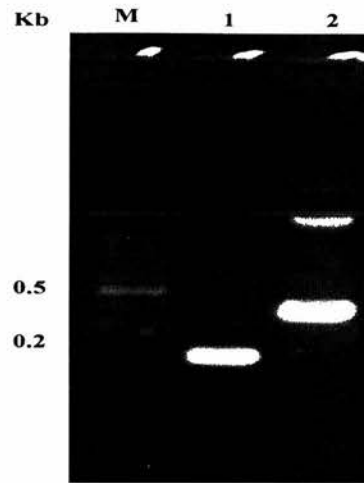


Figure 23: *T. vivax* genomic DNA amplified with: lane 1–TVW (150 bp), lane 2 – TWJ (400 bp).

#### ➤ **Screening of cattle for *T. congolense* by PCR amplification**

Since there was no sensitive single primer set that could detect all the four types of *T. congolense* in one PCR amplification reaction at the time of the study, cattle were screened with four sets of type-specific primers. The TCS1 and TCS2 primers were used to amplify the Savannah type of *T. congolense*. These primers, Table 9, target repetitive DNA elements unique to a previously described *T. congolense* Savannah type, on the basis of isoenzyme types (Young and Godfrey, 1983; Gibson, *et al.*, 1987; Gibson, Dukes and Gashumba, 1988; Majiwa and Otieno, 1990). The PCR cocktail was prepared with 0.2 $\mu$ M of both forward and reverse primers and genomic *Taq* polymerase (Sigma). The DNA was amplified as follows: step 1 - 94°C for 3 minutes, step 2 - 94°C for 1 minute; step 3 - 55°C for 2 minutes; step 4 - 72°C for 2 minutes. The amplification was cycled 30 times from step 2 and the final extension done at 72°C for 5 minutes. The samples which had the expected band sizes of 316 base pairs as shown in Figure 24 (a) lane 1 were regarded as positive for *T. congolense* Savannah type.

The TCK1 and TCK2 primers were used to amplify the Kilifi type of *T. congolense*. These primers target *T. congolense* (Kilifi type) and give a product of 294 base pairs, (Majiwa, *et al.*, 1994). The primers, Table 9, target repetitive DNA elements unique to a previously described, on the basis of the unusual iso-enzyme variant types, as *T. congolense* Kilifi type from the Kenyan coast, (Gashumba, 1986; Knowles, *et al.*, 1988; Majiwa, *et al.*, 1985). The PCR cocktail was prepared with 0.2 $\mu$ M of both forward and reverse primers and genomic *Taq* polymerase (Sigma). The DNA was amplified according to the protocol for TCS primers. The samples which had the expected band size of 294 bp as shown in Figure 24 lane 2 were regarded as positive for *T. congolense* Kilifi type.

The TCT1 and TCT2 primers were used to amplify Tsavo type of *T. congolense*. These primers Table 9, target highly repetitive tandemly arranged satellite DNA elements that are unique *T. congolense* Tsavo type and give a product of 400 bp. The Tsavo type of *T. congolense* was originally isolated from a tsetse fly caught in the Tsavo National Park, Kenya, (Majiwa, *et al.*, 1993). The PCR cocktail was prepared with 0.4 $\mu$ M of both forward and reverse primers and genomic *Taq* polymerase (Sigma). The DNA was amplified as follows: step 1 at 94°C for 3 minutes, step 2 at 94°C for 1 minute; step 3 at 60°C for 2 minutes; step 4 at 74°C for 30 seconds. The amplification was cycled 30 times from step 2 and the final extension done at 74°C for 5 minutes. The samples that had the expected band size of 400pb, as shown in Figure 24 lane 3 were regarded as positive for *T. congolense* Tsavo type.

The TCF1 and TCF2 primers were used to amplify Forest type of *T. congolense*. These, Table 9, target repetitive DNA elements unique to a previously described as *T. congolense* Forest type, on the basis of iso-enzyme typing, (Young and Godfrey, 1983; Gibson, Dukes and Gashumba, 1988; Gashumba, *et al.*, 1988; Masiga, *et al.*, 1992),. The PCR cocktail was prepared with 0.2 $\mu$ M of both forward and reverse primers. The DNA was amplified as follows: step 1 at 94°C for 3 minutes, step 2 at 94°C for 1 minute; step 3 at 55°C for 2 minutes; step 4 at 72°C for 2 minutes. The amplification was cycled 30 times from step 2

and the final extension done at 72°C for 5 minutes. Following electrophoresis, the samples which had the expected band size of 350 pb, as shown in Figure 25 lane 3 were regarded as positive for *T. congolense* Forest type.

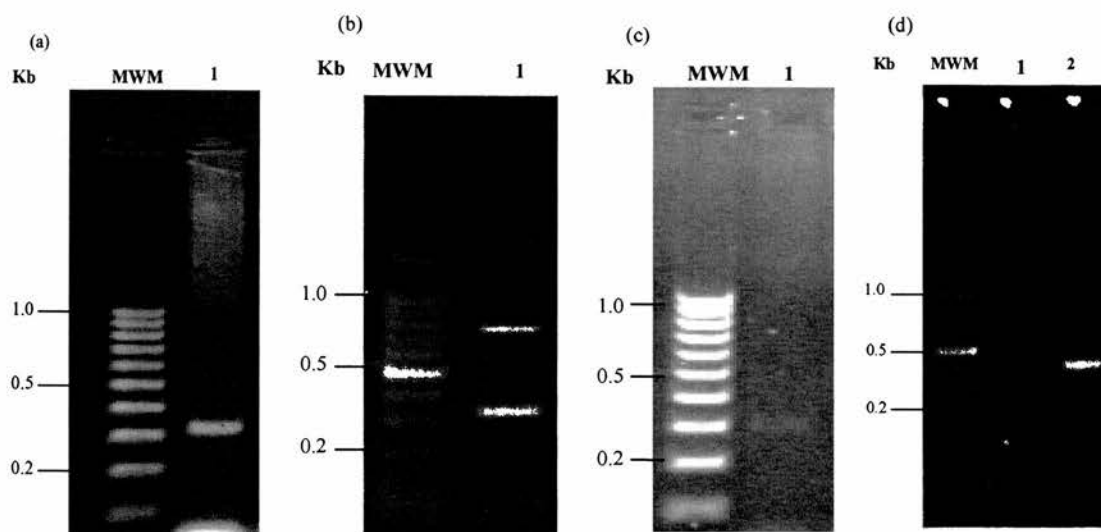


Figure 24: *T. congolense* genomic DNA amplified with primers targeting (a) Savannah type 316bp - lane 1, (b) Forest type 350bp - lane 1, (c) Kilifi type 294 bp - lane 1 and (d) Tsavo type 400bp - lane 2.

#### 3.2.2.4 Screening of cattle for tick-borne parasites by molecular techniques

PCR screening of cattle for *T. parva* was done with the p104 nested primers. The *T. parva* p104 antigen is present in the apical complex, localized in the rhoptries, the secretory organelles and is expressed specifically in sporozoites (Iams *et al.*, 1990b). It consists of a 104kDa molecule encoded by a 2772bp long genomic open reading frame (ORF) with no evidence of introns. A 4.5 Kb genomic cDNA clone containing the p104 gene had been sequenced and the sequence alignments compared between three isolates. The sequence is strongly conserved, except for point mutations at 22 places dispersed over 750bp within the C-terminal end. Primer sequences from an invariant region within this gene which are specific for *T. parva* have been used to determine the persistence of *T. parva* DNA in infected and recovered cattle following experimental infection (Skilton *et al.*, 2002). These

primers, Table 10, referred to as p104 external primers, generate a specific product of 496bp with *T. parva* DNA (Skilton *et al.*, 2002).

Table 10: The primers used to amplify p104 gene of *T. parva*

<b><u>Primer</u></b>	<b><u>Sequence 5' → 3'</u></b>
<b>p104 forward (external)</b>	5'-ATT TAA GGA ACC TGA CGT GAC TGC-3'
<b>p104 reverse (external)</b>	5'-TAA GAT GCC GAC TAT TAA TGA CAC C-3'
<b>p104 nested forward (internal)</b>	5'-GGC CAA GGT CTC CTT CAG ATT ACG-3'
<b>p104 nested reverse (internal)</b>	5'-TGG GTG TGT TTC CTC GTC ATC TGC-3'

The PCR reaction mixture was made as follows: 2.5µl of 10x PCR buffer consisting of 200mM KCL, 40mM Tris-HCl [pH 9.0], 0.4% Triton X-100; 6mM MgCl<sub>2</sub>, 200µM of each dNTP; 50ng of each p104 forward and reverse primer and 1.25 units of *Taq* DNA polymerase (Promega, Madison, WI, USA). The master mix was made sufficient for the number of samples to be amplified and aliquoted into 23µl. To each of the aliquots 2µl of purified DNA from each of the animals were added. To monitor for the occurrence of false-positive and false negative PCR results, negative control DNA purified from uninfected cattle blood was included and a positive control purified DNA from *T. parva* infected blood at 2% parasitaemia were used. Both positive and negative controls were included each time the PCR was performed. The reactions were cycled in a MJ-PTC 100 thermal cycler (MJ Research Waltham, MA, USA), as follows: 94°C for 1 minute followed by 40 cycles of 94°C for 1 minute, 60°C for 1 minutes and 72°C for 1 minute, plus an additional 9 minutes at 72°C for extension, after the last cycle. The samples containing bands that were similar in size to the expected band in the positive control samples, 496bp, were regarded as *T. parva* positive.

In order to improve on the sensitivity of the PCR the internal were used in the nested PCR as internal primers, Table 10. These primers result in a product of 278bp, Figure 25. The sensitivity of this nested PCR has been shown to be capable of picking parasitaemia of



approximately 50 or 37 parasites/ $\mu$ l and was found to be more sensitive in detecting *T. parva* carrier state compared to the reverse line blot, (42.7% vs. 10.6%), (Odongo, 2004).

The PCR reaction mixture was done as follows: 2.5 $\mu$ l of 10x PCR buffer was mixed with 1.5 $\mu$ l of 25mM MgCl<sub>2</sub>, 0.25 $\mu$ l of 25mM dNTP, 0.25 $\mu$ l of 100ng forward and reverse primers, 0.125 $\mu$ l of 5U/ $\mu$ l Promega *Taq* polymerase enzyme and 19.125 $\mu$ l of double distilled sterile water. The master mix was made sufficient for the number of samples to be amplified and aliquoted into 24 $\mu$ l. To each of the aliquots 1 $\mu$ l of amplified PCR DNA product of the first PCR with external primers, from each of the animals, was added followed with a drop of mineral oil. Amplification was done according to the following program: 94°C to denature the DNA template, 55°C to anneal the primers to the template, 72°C to extend the products and the cycling was repeated 29 times. The resulting internal band was approximately 278bp, Figure 25, lane 1.

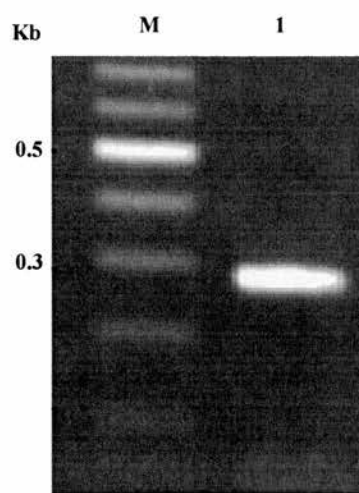


Figure 25: *Theileria parva* piroplasms p104 DNA product from nested PCR amplification.

➤ **Detection of other tick-borne parasites in cattle by Reverse Line Blot (RLB)**

Mixed infections of tick-borne haemoparasites and other species of *Theileria* that are of low pathogenicity or avirulence frequently occur under field conditions and need to be distinguished from the pathogenic *T. parva*. Hence a multiple detection test such as the

reverse line blot hybridization could be a valuable tool in the simultaneous detection of such infections (Georges *et al.*, 2001; Almeria *et al.*, 2001). The RLB technique was used with additional probes for the detection of *Theileria*, *Babesia*, *Anaplasma* and *Ehrlichia* species, Table 11, which frequently occur simultaneously in carrier animals. The objective was to show the presence of several haemoparasites in cattle exposed to ticks in field conditions in the 4 study villages of Tororo district. The RLB assay is a versatile diagnostic tool and can be used to simultaneously detect and differentiate haemoparasites in blood or ticks infecting a single animal (Gubbels *et al.* 1999). The essence of the technique is based in simultaneous PCR amplification of related microorganism species; the *Ehrlichia* species on one side, and the *Theileria/Babesia* species on the other. The individual species are then differentiated by hybridization of the PCR products to species-specific oligonucleotide probes, Table 12, immobilized on a membrane, using a line-blotter apparatus, thus multiple sequences can be analyzed against multiple probes to enable simultaneous detection of parasites from an individual animal or tick species.

Table 11: Possible co-infecting tick-borne pathogens other than *Theileria parva* used as a source of DNA in this study. All parasites are cattle derived except where indicated.

<u>Parasite species</u>	<u>Stage</u>	<u>Country</u>	<u>Reference</u>
<i>Theileria annulata</i>	Piroplasm	Sudan	Melrose <i>et al.</i> , 1984).
<i>Theileria mutans</i> (Intona)	Piroplasm	Kenya	Morzaria <i>et al.</i> , (1990).
<i>Theileria taurotragi</i> (R6) <sup>§</sup>	Schizont	Kenya	Bishop <i>et al.</i> , (1992).
<i>Theileria velifera</i>	Piroplasm	Tanzania	Gubbels <i>et al.</i> , (1999).
<i>Theileria buffeli</i> Marula	Piroplasm	Kenya	Ngumi <i>et al.</i> , (1992).
<i>Theileria lestoquardi</i> <sup>a</sup>	Piroplasm	Sudan	Skilton <i>et al.</i> , (2002).
<i>Theileria buffalo</i> species	Schizont	Kenya	Bishop & Odongo <sup>b</sup>
<i>Babesia bigemina</i>	Merozoite	Kenya	Katende <i>et al.</i> , (1998).
<i>Babesia bovis</i>	Merozoite	Australia	W. Jorgensen <sup>c</sup>
<i>Anaplasma marginale</i>	Merozoite	Kenya	Katende <i>et al.</i> , (1998).
<i>Anaplasma centrale</i>	Merozoite	Brazil	C. Madruga <sup>d</sup>
<i>Ehrlichia canis</i>	Blood	USA	Gubbels <i>et al.</i> , (1999).
<i>Cowdria ruminantium</i>	Endothelium	Senegal	Gubbels <i>et al.</i> , (1999).

Isolated from African buffalo (*Syncerus caffer*).

<sup>a</sup> Isolated from Sheep

<sup>b</sup> Different from *T. parva* based on molecular characterisation using DNA probes, but causing clinical disease in cattle. This isolate was obtained from an animal that grazed together with a herd of wild buffalos. The animal was suspected to have received a buffalo-type of parasite challenge (Bishop & Odongo, unpublished data).

<sup>c</sup> Tick Research Centre, Waco, Australia.

<sup>d</sup> Institute of Agriculture and Livestock Research, Embrapa, Brazil.

PCR screening of cattle for tick-borne parasites was done with primers targeting various *Theileria* species, (*T. parva*, *T. annulata*, *T. taurotragi*, *T. mutans*, *T. velifera*, *T. buffeli*, *T. buffalo* sp); *B. bigemina* and the *Ehrlichia* group, (*A. marginale*, *A. centrale*, *B. bovis* or *E. ruminantium*). Two sets of primers, Table 12, were separately used to amplify the 460-520bp fragment of the 18S and 16S SSU rRNA spanning the V4 variable region of *Theileria/Babesia* and *Erhlichia* group. The primers were obtained from MWG (Germany). All PCR reagents were from Promega (Madison, Wisconsin, USA). Reaction conditions in 25µl volume were as follows; 1x PCR buffer, 1.5mM MgCl<sub>2</sub>, 200µM each deoxynucleoside triphosphate (dNTP), 0.1 units of Uracil DNA glycosylase (UDG), 2.5 units of *Taq*

polymerase, 100pmol/μl of each of the *Theileria/Babesia* primers and 2.5μl of purified DNA samples. PCR primers for the *Ehrlichia* species were diluted to 20pmols/μl each, immediately before being used.

The mixture was incubated for 3 minutes at 37°C to promote UDG activity, followed by 10 minutes incubation at 94°C to inactivate the UDG. Uracil DNA glycosylase acts by excising deoxyuracil from dU-containing DNA into acid soluble counts, by cleaving the *N*-glycosidic bond between the uracil base and the sugar phosphate backbone. The end effect of UDG is the elimination of carry-over contamination of PCR products to subsequent reactions. To minimize non-specific amplification, a touchdown PCR program was used as follows: two cycles of 20s at 94°C, 30s at 67°C, and 30s at 72°C, and then two cycles with conditions identical to the previous cycles, but with an annealing temperature of 65°C. During the subsequent two cycle sets, the annealing temperatures were lowered by 2°C down to 59°C. An additional step of 40 cycles, each 20s at 94°C, 30s at 57°C and 30s at 72°C, followed the touchdown program. The PCR was ended by an extra incubation for 1 h at 65°C to inactivate any traces of UDG and the samples were stored at -20°C until required.

Table 12 Sequences of RLB Oligonucleotide primers used for PCR (Gubbels *et al.*, 1999; Bekker *et al.*, 2002) (\*5' Biotin labeled.)

<u>Oligonucleotide</u>	<u>Sequence 5' → 3'</u>
<i>Theileria/Babesia</i> forward	5'-GACACAGGGAGGTAGTGACAAG-3'
<i>Theileria/Babesia</i> reverse*	5'-CTAAGAATTTACCTCTGACAGT-3'
<i>Ehrlichia</i> species forward	5'-GGAATTCAGAGTTGGATCMTGGYTCA-3'
<i>Ehrlichia</i> species reverse*	5'-CGGGATCCCGAGTTTGCCGGGACTTYTTCT-3'

The reactions were performed in an automated DNA thermal cycler (MJ Research), with an enabled hot lid. Five microlitres of the products were electrophoresed to verify that amplification took place while the rest of the amplification products were stored at 4°C until required for hybridization.

### **3.2.3 Gel Electrophoresis of PCR products**

The DNA from each PCR amplification product of in the screening of trypanosomes including positive and negative controls were electrophoresed in 1.5% agarose gel along with 100 base pair molecular weight markers, with 1 x TBE (89mM Tris-borate, 2mM EDTA, pH 8.3), buffer containing 0.05mg/L ethidium bromide. The amplified DNA band(s) were visualized under UV light and the image acquired onto a photo-imager. The positive bands were compared with those on the amplified positive trypanosome DNA controls and the right size determined by comparing against the molecular weight marker.

The PCR amplification products of tick-borne parasites were electrophoresed in 1.5% agarose gels (either from Sigma or Gibco BRL) in 1 x TAE pH 8.0 buffer (40mM Tris, 20mM Glacial acetic acid and 2mM EDTA), with 0.05mg/L ethidium bromide for 30 minutes, and then visualized on a 312 nm UV trans-illuminator (UVP Inc.). A digital image of the gel was captured from a digital gel reader (UVP Inc.) that was connected to a computer, and then printed from the connected thermal printer (Sony UPD 890).

### **3.2.4 Southern blot hybridization for RLB detection of tick borne parasites**

In the second step of the reverse line blot, the PCR products are hybridized to a blot onto which known *Anaplasma*, *Ehrlichia*, *Theileria* and *Babesia* species-specific oligonucleotide probes, Table 13, have been bound. These primer probes are designed to species-specific sequences from the hyper-variable region that has been amplified in the first step, have been covalently linked through a 5' terminal amino linker. All the species-specific oligonucleotides contained an N-terminal N-(trifluoroacetamidohexyl-cyanoethyl),*N,N*-diisopropyl phosphoramidite [TFA]-C<sub>6</sub> amino linker (MWG-Germany).

They were diluted to the optimized concentrations ranging from 50 - 800pmol in 150µl of 500mM NaHCO<sub>3</sub>, pH 8.4.

Table 13: The sequences of oligonucleotide probes hybridized onto the Reverse line blot membrane.

Oligonucleotide probe	Sequence 5' → 3'	Reference
<i>Theileria &amp; Babesia</i> catch-all	TAA TGG TTA ATA GGA RCR GTT G	Gubbels <i>et al.</i> , (1999)
<i>Theileria annulata</i>	CCT CTG GGG TCT GTG CA	Georges <i>et al.</i> , (2001)
<i>Theileria parva</i>	TTC GGG GTC TCT GCA TGT	Gubbels <i>et al.</i> , (1999)
<i>Theileria mutans</i>	CTT GCG TCT CCG AAT GTT	Gubbels <i>et al.</i> , (1999)
<i>Theileria taurotragi</i>	TCT TGG CAC GTG GCT TTT	Gubbels <i>et al.</i> , (1999)
<i>Theileria velifera</i>	CCT ATT CTC CTT TAC GAG T	Gubbels <i>et al.</i> , (1999)
<i>Theileria buffeli/orientalis</i>	GGC TTA TTT CGC WTT GAT TTT	Gubbels <i>et al.</i> , (1999)
<i>Theileria buffalo</i> sp	CAG ACG GAG TTT ACT TTG T	unpublished
<i>Babesia bigemina</i>	CGT TTT TTC CCT TTT GTT GG	Gubbels <i>et al.</i> , (1999)
<i>Babesia bovis</i>	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels <i>et al.</i> , (1999)
<i>Ehrlichia</i> catch-all	GGG GGA AAG ATT TAT CGC TA	Bekker <i>et al.</i> , (2002)
<i>Ehrlichia ruminantium</i>	AGT ATC TGT TAG TGG CAG	Gubbels <i>et al.</i> , (1999)
<i>Anaplasma centrale</i>	GGA CCA TAC GCG CAG CTT	Georges <i>et al.</i> , (2001)
<i>Anaplasma marginale</i>	GAC CGT ATA CGC AGC TTG	Bekker <i>et al.</i> , (2002)

### 3.2.5 RLB hybridization

A biodyne-C blotting membrane (Pall Gelman Bio-support, Michigan), was activated by incubation in 10ml of freshly prepared 16% (w/v) solution of 1-ethyl-3 [3-dimethylaminsopropyl] carbodiimine (EDAC) in ddH<sub>2</sub>O water for 10 minutes. The membrane was then rinsed in water and placed in a clean mini-blotter system (Immunetics, Cambridge, MA, USA), and the slots filled with 150µl of the diluted oligonucleotide solutions. After incubation for one minute at room temperature, the oligonucleotide solutions were aspirated, and the membrane removed from the mini-blotter which was then incubated at room temperature in 100ml of 100mM NaOH for 10 minutes and finally



washed with gentle shaking in 100ml of 2x SSPE/ 0.1% SDS w/v for 5 minutes at 60°C (20X SSPE pH 7.4 = 3 M NaCl, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM EDTA). In filling the slots with the PCR products, the membrane was replaced in the mini-blotter with the slots perpendicular to the orientation of the previously bound species-specific oligonucleotides lines. To carry out a reverse line blot hybridization, a volume of 10µl of each *Ehrlichia* and *Theileria/Babesia* PCR products were combined and mixed with 130µl of 2x SSPE/ 0.1% SDS, heated for 10minutes at 100°C, and cooled immediately on ice. Denatured PCR samples were applied into the slots, and hybridization was performed by incubation for 1 h at 42°C. PCR products were aspirated, and the blot was removed from the mini-blotter apparatus and washed twice in 100ml of 2x SSPE/ 0.5% SDS for 10 minutes at 52°C with shaking. Subsequently the membrane was incubated in 10 ml of 1:4000-diluted peroxidase - labeled streptavidin (Boehringer, Mannheim, Germany) in 2x SSPE/ 0.5% SDS for 30 minutes at 42°C.

A biotin label attached to a PCR primer allows for the visualization through the presence of biotin on the membrane that is detected by incubation with streptavidin ligand conjugated to horse-radish peroxidase. The membrane was then washed twice in 100ml of 2x SSPE/ 0.5% SDS for 10 minutes at 42°C with shaking. After two further washes of 5 minutes each time at room temperature in 100ml of 2x SSPE, the blot was incubated for 1 minute in 10 ml of substrate ECL detection fluid (Amersham, Little Chalfont, Buckinghamshire, UK) then exposed to an ECL hyper-film (Amersham) for 10 - 20 minutes. The hybridized PCR products can then be visualized using chemiluminescence after stringent washing to remove unbound PCR products. Incubation of the blot with the peroxidase substrate results in a light producing reaction, which can be detected on an x-ray film Figure 26. The x-ray hyper-film was processed in an automatic x-ray film developer to visualize the hybridization signals. Spots occur on the developed film at the sites where species-specific oligonucleotide probes and PCR-products hybridized. The identity of the microorganism(s) present in the sample can be readily deduced relative to a positive control sample, Figure 26. The process is illustrated schematically in Figure 27.

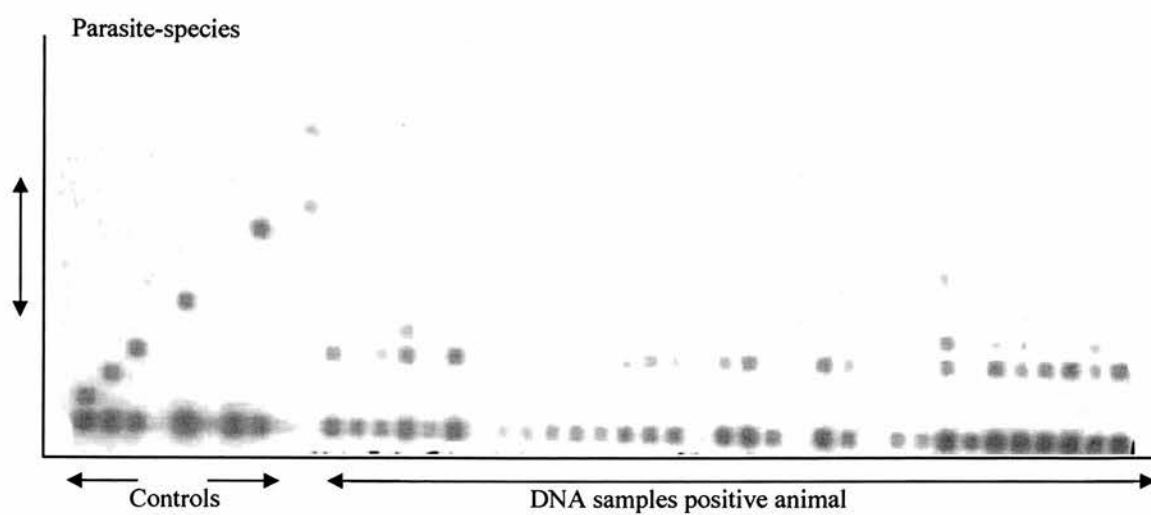


Figure 26: Autoradiograph showing hybridization signals from controls and some positive samples

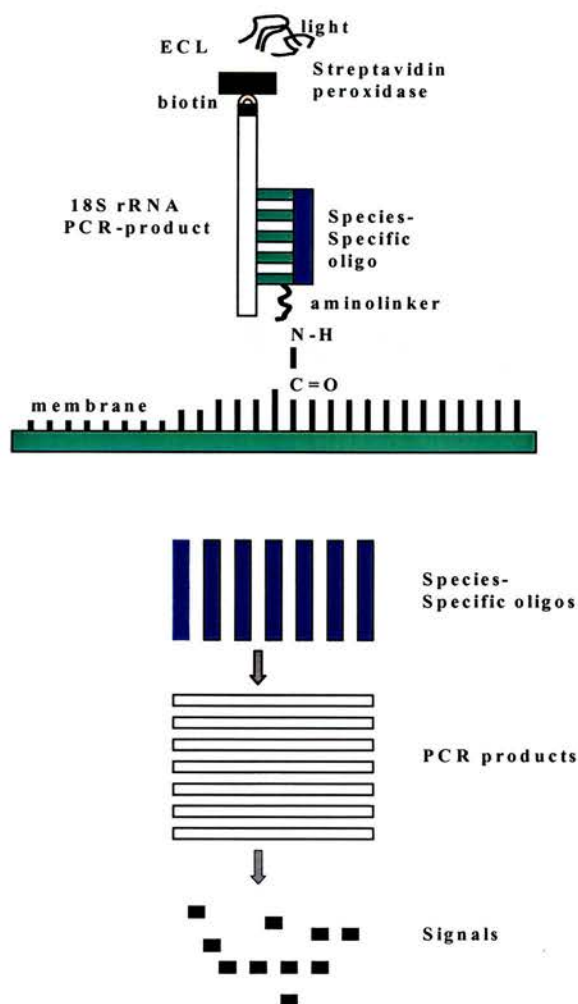


Figure 27: Schematic presentation of the reverse line blot (RLB) assay.

### 3.2.6 Stripping of the hybridization membrane

Since the labeled membrane can be used 20 times, all hybridized PCR products were stripped from the membrane after each use, by two washes at 80°C for 30 minutes each in 1% SDS while shaking. The membrane was then rinsed in 20mM EDTA pH 8.0 and stored in fresh pH 8.0 20mM EDTA solution at 4°C for re-use. Under these conditions the membrane remained active and was reused to a maximum of 20 times without loss of sensitivity of the species-bound oligonucleotides.

## CHAPTER FOUR

### 4 BASELINE PREVALENCE OF VECTOR BORNE PARASITES IN CATTLE

#### 4.1 INTRODUCTION

Epidemiological studies of vector borne parasites that infect cattle in Uganda have traditionally been conducted using data collected from microscopic screening of animals. The data obtained by microscopy has generally been accepted and used in describing disease incidence and prevalence in many locations including SE Uganda, (Magona, *et al.*, 2003; Waiswa, *et al.*, 2003). The method is quick and relatively inexpensive and provides both qualitative (the organisms can be visualized and identified) and quantitative estimation (the density of infection can be quantified) of the disease burden.

However, the method lacks sensitivity in detecting low parasitaemia in cattle, a common characteristic in naturally infected animals. The technique also lacks the specificity required to characterize various parasite species, sub-species and strains infecting cattle, thus it cannot distinguish pathogenic from non-pathogenic species of parasites. For example, it is not possible to definitively distinguish some species of vector-borne parasites such as *T. parva* from *T. mutans* (Ogden, *et al.*, 2003) or differentiate the sub-species of *T. brucei* (Welburn, *et al.*, 2002) by microscopy, even by well trained and experienced personnel. The ability to differentiate the parasites to these levels is important in deciding on treatment and control measures to be taken against infections in any particular area and is also important for epidemiological investigations.

Since the advent of molecular diagnostic methods a number of markers have been identified that help target specific species and sub-species of parasites. These molecular markers allow for characterization of individual species of parasites with unique genomic DNA sequences. The techniques are also much more sensitive than the traditional microscopy since many of the markers target DNA molecules that occur in multiple repeats and copy numbers in the genome of an organism. These methods include the hybridization,

PCR and a combination of both hybridization and polymerase chain reaction (PCR) amplification. Whereas hybridization techniques require large amounts of purified DNA or RNA and are therefore not suitable for diagnostic purposes, the use of PCR amplification has been extensively used in laboratory experiments and have been evaluated for field studies (Moser, *et al.*, 1989; Masiga, *et al.*, 1992; Majiwa, *et al.*, 1994; Long *et al.* 1995; Masake, *et al.*, 1997; Gubbels *et al.* 1999; Clausen, *et al.*, 1998 Almeria *et al.*, 2001 Georges *et al.*, 2001; Skilton *et al.* 2002).

The kappa statistic employed in determining the diagnostic agreement between microscopy and PCR amplification in this Chapter, has been used in a wide range of studies. For example, it has been used in the determination of agreement between sampling methods and time of sampling for the detection of *Salmonella* in Danish infected chicken flocks, (Gradel, *et al.*, 2002). It has also been used in determining the interobserver variability associated with MIB-1 labeling index as a potential prognostic marker for patients with primary brain tumours, (Grzybicki, *et al.*, 2001). The method has also been used in estimation of diagnostic agreement between a clinical examination and electrophysiological tests such as quantitative computerized tremor analysis in essential tremor (ET) (Louis and Pullman, 2001). In particular, Greiner *et al.* (1997) used the the method in evaluation and comparison of antibody ELISAs for the serodiagnosis of bovine trypanosomiasis in Mukono County, SE Uganda.

The prevalence and incidence of vector transmitted parasites in cattle has traditionally been studied by using microscopy and immunological diagnostic methods such as ELISA. However, these methods are either insensitive, non specific or they do not discriminate between past and present infections. In SE Uganda previous studies have shown that the prevalence of trypanosomes in cattle was as high as 7.1%, *Theileria* species, 33.7% and *Anaplasma marginale* was 13.3% (Magona and Mayende, 2002). Other studies have indicated that the prevalence of trypanosomes in cattle under communal grazing system was 25% (Okuna, *et al.*, 1996) while the prevalence of theileriosis, anaplasmosis and

babebiosis had been found to be 48%, 36% and 16% respectively (Anon, 1996). These studies had all used microscopy to examine the samples from cattle.

The use of PCR amplification which has been demonstrated in the previous Chapter to be superior to microscopy in terms of sensitivity and specificity and can show only existing infections, allows the evaluation of the prevalence and incidence of individual trypanosome species affecting cattle. In Uganda, epidemiological studies have been conducted on a small scale in cattle using PCR amplification, in the peri-urban dairy production systems, located near the capital Kampala (Clausen, *et al.*, 1999). The baseline prevalence was determined in order to assess the impact of the vector transmitted diseases in the area prior to prophylactic drug treatment.

#### **4.1.1 Objectives of the study in the Chapter**

The primary objective of this Chapter was to evaluate the diagnostic performance of microscopic examination of cattle infected with trypanosomes and tick-borne parasites against the molecular methods. The purpose of the evaluation was to help in deciding the most appropriate method, in terms of sensitivity and specificity in parasite detection. The most appropriate method was to be used for screening cattle for disease prevalence and the drug intervention in the subsequent study. Blood and buffy coat samples from cattle were screened by microscopy for trypanosome species and tick-borne parasites as detailed in Chapter 3. The use of one step PCR for the detection of *Theileria parva* piroplasms in which only internal primers are used, was also evaluated against a nested PCR where both internal and external primers were used. The samples were also screened for the same organisms by the reverse line blot hybridization (RLB), also detailed in Chapter 3, to detect tick-borne parasites.

The second objective of this Chapter was to establish the baseline prevalence of the vector transmitted parasites in cattle from the villages of Tororo and Busia districts prior to drug intervention. In order to achieve this, both blood and buffy coat samples from cattle were screened as has been described in Chapter 3.



## 4.2 MATERIALS AND METHODS

Baseline blood samples, collected from a total of 639 animals, 319 from Tororo and 320 from Busia villages, were examined for *T. brucei*, *T. congolense*, *T. vivax* and tick-borne parasites by both microscopic examination of the buffy coat preparations and Giemsa stained thick and thin blood smears and molecular methods as described in Chapter 3.

### 4.2.1 Evaluation of the diagnostic techniques used for detection of vector-borne parasites

The diagnostic performance of microscopy in detecting the three trypanosome species in cattle was compared with PCR amplification. Cohen's kappa statistic was used to determine the diagnostic agreement between microscopy and PCR amplification as it gives an indication for the degree of agreement between two or more methods (Woodward, 1999). The kappa values lie between 0 and 1, where 1 means a perfect agreement between tests and 0 means that the association is no better than expected from chance alone, (Woodward, 1999, Picozzi, *et al.*, 2002). A total of 639 cases from the villages of both Tororo and Busia districts were used for the evaluation of the performance of microscopy and PCR in diagnosing trypanosomes.

A total of 319 cases from the baseline samples in the Tororo villages were used in the evaluation of the diagnostic performance of single vs. nested PCR amplification of the *Theileria parva* p104 gene. Diagnostic performance of internal primers targeting the p104 gene of *Theileria parva* alone in detecting *T. parva* was evaluated against the use of both internal and external primers in a nested PCR amplification as gold standard by the Cohen's kappa statistic protocol. The analyses for the agreement in performance of single p104 PCR amplification with internal primers against nested PCR were done as with the trypanosomes infection in cattle.

The sensitivity of any technique is defined as the number of positive observations (detections) common to the techniques being evaluated divided by the combined positives from all the techniques. The specificity on the other hand is defined as the number of

negative observations common to the techniques being compared divided by the combined negatives from both techniques (Woodward, 1999, Magona *et al.*, 2003). The kappa value was calculated by the formula:  $((A-E)/(N-E))$ , where A is the sum of agreements defined as the sum of positives and negatives common to both techniques, E is the sum of expected occurrences defined as the sum of all positives divided by N plus the sum of all negatives divided by N,  $((\sum \text{Positives})/N) + ((\sum \text{Negatives})/N)$  and N is the number of animals examined by both methods. In summary:

$$\text{Kappa value} = ((A-E)/(N-E))$$

Where  $A = \sum \text{All positives and negatives common to both techniques}$

$$E = ((\sum \text{Positives})/N) + ((\sum \text{Negatives})/N)$$

And N = the total number of animals examined by both techniques.

#### **4.2.2 Determination of the prevalence of trypanosome species in cattle**

The prevalence of various trypanosome species was determined from the animals which tested positive by microscopy and PCR amplification methods from Tororo and Busia districts. Trypanosome species were detected in cattle by microscopy and PCR amplification as has been described in Chapter 3. The data from each district was treated separately in order to determine whether there were any variations between districts. The data was then combined to give the overall prevalence in both districts. Comparisons of the prevalence were made between the data obtained by microscopy and PCR amplification using the Chi-square statistical analysis.

#### **4.2.3 Determination of the prevalence of tick-borne parasites in cattle**

Baseline blood samples from a total of 319 animals from the four villages of Tororo district were screened for *Theileria*, *Babesia*, *Anaplasma* and *Cowdria* species by both microscopy and molecular methods. The prevalence of different *Theileria* species was determined by both the nested PCR amplification and the reverse line blot hybridization assay (RLB) methods as detailed in Chapter 3. The prevalence of *Theileria*, *Babesia* and *Anaplasma* was determined by microscopic examination of thick and thin smears stained with Giemsa' stain.

## 4.3 RESULTS

### 4.3.1 Evaluation of the diagnostic performance of microscopy on trypanosomes

The results of the analysis of the diagnostic agreement of microscopy with PCR amplification for diagnosing *T. vivax*, *T. congolense* and *T. brucei* in cattle is shown in Tables 13 – 16. The sensitivities, specificities and kapp values are summarized in Table 17. The results of the diagnostic performance of PCR amplification of the *T. parva* p104 gene with internal and nested PCR amplification is shown in Table 18.

Out of a total of 639 cattle from both Tororo and Busia villages examined for *T. vivax* by both microscopy and PCR amplification, 5 animals (0.8%) were found to be positive for *T. vivax* by both methods, 18 animals (2.8%) were positive by microscopy but negative with PCR, 97 animals (15.2%) were positive by PCR but negative by microscopy while 537 animals (81.2%) were found negative by both methods. A total of 23 animals (4%) were positive by microscopy while 102 (16%) were positive by PCR amplification, Table 14.

Table 14: Number of animals tested for *T. vivax* in cattle by both microscopy and PCR amplification.

	Positive by PCR	Negative by PCR	Total
Positive by microscopy	5	18	23
Negative by microscopy	97	519	616
Total	102	537	639

The sensitivity of microscopy in detecting *T. vivax* was found to be 4.9% while the specificity was 96% against PCR amplification. The diagnostic predictive value showed that the probability of microscopy detecting positive *T. vivax* in cattle was 0.2 while the probability of detecting *T. vivax* negative cattle was 0.8. The kappa value was determined to be 0.02, Table 17

Out of a total of 636 cattle from both Tororo and Busia villages examined for *T. congolense* by both microscopy and PCR amplification, 2 animals (0.3%) were found to be positive for *T. congolense* by both methods, 9 animals (1%) were positive by microscopy but negative by PCR, 104 (16.4%) were positive with PCR but negative by microscopy while 522 (82.1%) were found negative by both methods. A total of 11 animals, (2%) were positive with microscopy while 106 (17%) were positive by PCR amplification, Table 15.

Table 15: Number of animals tested for *T. congolense* in cattle by both microscopy and PCR amplification

	Positive by PCR	Negative by PCR	Total
Positive by microscopy	2	9	11
Negative by microscopy	104	521	625
Total	106	530	636

The sensitivity of microscopy in detecting *T. congolense* was found to be 1.7% while the specificity was 98.2% against PCR amplification. The diagnostic predictive value showed that the probability of microscopy detecting positive *T. congolense* in cattle was 0.18 while the probability of detecting *T. congolense* negative cattle was 0.17. The Kappa value was determined to be 0.003, Table 17.

Out of a total of 638 cattle from both Tororo and Busia villages examined for *T. brucei* by both microscopy and PCR amplification, no animal was found to be positive with *T. brucei* by both methods, 10 (1.2%) were positive with microscopy but negative with PCR, 75 (11.8%) were positive by PCR but negative by microscopy while 553 (86.7%) were found negative by both methods (Table 16). The sensitivity, specificity as well as the kappa value for microscopic detection of *T. brucei* in cattle were 16%, 98.4% and -0.028, respectively, Table 17. The diagnostic predictive values could not be determined since there were no positive animals detected by both methods.

Table 16: Number of animals tested for *T. brucei* in cattle by both microscopy and PCR amplification.

	Positive by PCR	Negative by PCR	Total
Positive by microscopy	0	10	10
Negative by microscopy	75	553	628
Total	75	563	638

When the analysis was done on any of the species detectable by both methods, the sensitivity of microscopy against PCR amplification was found to be 4.9%, the specificity was 93.7% while the kappa value was -0.02.

Table 17: Summary of sensitivity, specificity and kappa values of microscopy against PCR amplification

	<i>T. brucei</i>	<i>T. congolense</i>	<i>T. vivax</i>	Any species
Sensitivity	1.6%	1.7%	3.6%	4.9%
Specificity	98.4%	98.2%	96.4%	93.7%
Kappa value	-0.028	0.003	0.02	-0.02

#### 4.3.2 Performance of p104 PCR internal primers in detecting *T. parva* in cattle

The sensitivity of PCR amplification with internal primers was found to be 5% while the specificity was 89% with a predictive value probability for detecting p104 positives was 0.18 and for detecting p104 negatives was 0.93. The Kappa value was 0.12, Table 18.

Table 18: Number of animals tested for *T. parva* in cattle by both internal primers (IP) alone and in a nested PCR (N-PCR) amplification.

	Positive by N- PCR	Negative by N- PCR	Total
Positive by IP	7	32	39
Negative by IP	20	260	280
Total	27	292	319
Sensitivity	5%		
Specificity	89%		
Kappa value	0.12		

### 4.3.3 Baseline prevalence of vector borne parasites in cattle from the villages of Tororo and Busia

#### 4.3.3.1 Baseline prevalence of trypanosome species circulating in cattle from the villages of Tororo and Busia districts.

The prevalence of the three trypanosome species, *T. brucei*, *T. congolense* and *T. vivax*, circulating in cattle in the villages of Tororo and Busia districts was determined by microscopy and PCR amplification of the buffy coat samples on FTA filter cards. The prevalence of the three species in cattle is shown in Tables 19-21 while the prevalence of *T.b. rhodesiense* is shown in Table 22.

The results indicate that the prevalence of *T. brucei*, *T. congolense* and *T. vivax*, in the 319 cattle as determined by microscopy was 2.8%, 1.3% and 4.7% respectively. The corresponding prevalence in the same animals as determined by PCR amplification was 7.5%, 13% and 18.5%. The differences in prevalences between microscopy and PCR were highly significant,  $\chi^2 = 6.17$  for *T. brucei*, 30 for *T. congolense* and 28 for *T. vivax* and  $p < 0.05$  in all the groups, Table 19.



Table 19: The prevalence of trypanosome species in cattle from Tororo district (n = 319)

<u>Trypanosome species</u>	<u>Microscopy</u>	<u>PCR</u>	<i>Ratio (Microscopy/PCR)</i>	<i>p-value</i>
	<i>Prevalence</i>	<i>Prevalence</i>		
<i>T. brucei</i>	2.8%	7.5%	1:3	0.01
<i>T. congolense</i>	1.3%	13%	1:10	< 0.001
<i>T. vivax</i>	4.7%	18.5%	1:4	< 0.001

The prevalence of trypanosome species in 320 cattle from the villages of Busia district as examined by microscopy was 0.3%, 2% and 2.5% for *T. brucei*, *T. congolense* and *T. vivax* respectively. The corresponding species prevalence as determined by PCR amplification in the same animals was 16%, 21% and 13%. The difference in prevalence between microscopy and PCR were highly significant,  $\chi^2 = 36$  for *T. brucei*, 52 for *T. congolense* and 25 for *T. vivax* and  $p < 0.001$  in all the groups Table 20.

Table 20: The prevalence of trypanosome species in cattle from Busia district (n = 320)

<u>Trypanosome species</u>	<u>Microscopy</u>	<u>PCR</u>	<i>Ratio (Microscopy/PCR)</i>	<i>p-value</i>
	<i>Prevalence</i>	<i>Prevalence</i>		
<i>T. brucei</i>	0.3%	16%	1:53	< 0.001
<i>T. congolense</i>	2%	21%	1:11	< 0.001
<i>T. vivax</i>	2.5%	13.4%	1:5	< 0.001

The overall prevalence for *T. brucei*, *T. congolense* and *T. vivax* in the 639 cattle examined in both Tororo and Busia villages by microscopy was 1.6%, 1.7% and 3.5% while the corresponding prevalence for the three trypanosome species as determined by PCR amplification was 12%, 17% and 16%, Table 16, a ratio of 1:8, 1:10 and 1:5.

Table 21: Overall prevalence of trypanosome species in cattle from Tororo and Busia districts (n = 639)

<u>Trypanosome species</u>	<u>Microscopy</u>		<u>PCR</u>	
	<b>This study</b>	<b>Previous study*</b>	<b>This study</b>	
	<i>Prevalence</i>	<i>Prevalence</i>	<i>Prevalence</i>	<i>Ratio(Microscopy/PCR)</i>
<i>T. brucei</i>	1.6%	1.59%	12%	1:8
<i>T. congolense</i>	1.7%	3.11%	17%	1:10
<i>T. vivax</i>	3.5%	1.73%	16%	1:5

\* (Ford, 1971)

#### 4.3.3.2 Trypanosome species proportions in cattle from the villages of Tororo and Busia

The results of the proportion of trypanosome species in positive cattle obtained from microscopic and PCR screening 319 cattle from Tororo and 320 from Busia districts are shown in Figures 28-33 below. The results indicate that out of the 28 cattle found to be infected with trypanosomes by microscopic examination, 9 animals 32% had *T. brucei*, 4 (14%), had *T. congolense* while 15 (54%) had *T. vivax* infections. The results also indicate that out of the positive animals detected by microscopy, there were none with mixed infections of *T. brucei* /*T. congolense*, (Tb/Tc), *T. congolense*/*T. vivax* (Tc/Tv) or mixed infection with all the three species (Tb/Tc/Tv), while 1 animal (4%) had *T. brucei*/*T. vivax* (Tb/Tv). The results indicate that out of the 124 cattle found to be infected with trypanosomes by PCR amplification, 24 animals (19%) had *T. brucei*, 42 (34%), had *T. congolense* while 58 (47%), had *T. vivax*. The results also show that out of all the positive animals 4 (3%), had mixed infections of *T. brucei*/*T. congolense*, 10 (8%) had *T. brucei*/*T. vivax* and 4 (3%) had *T. congolense*/*T. vivax* while 2 (1.6%) had mixed infections with all the three species Figure 28.

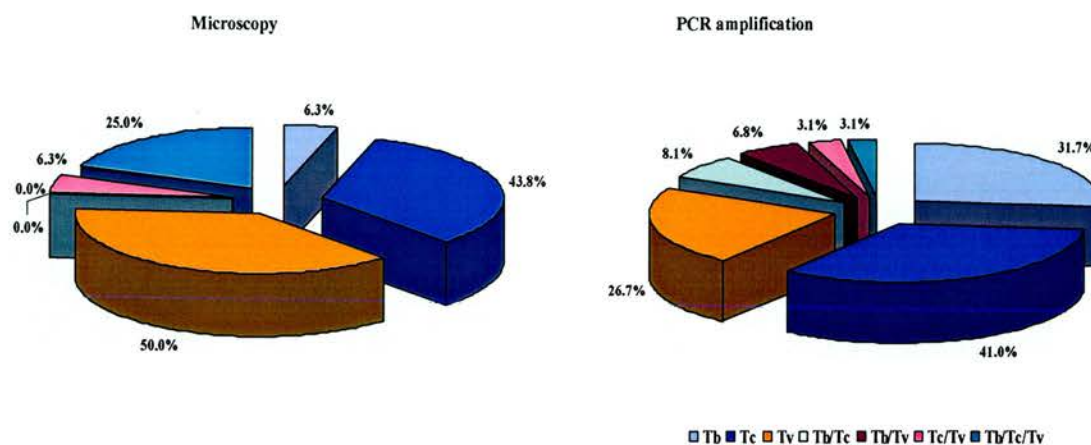


Figure 28: Proportion of animals with various trypanosome species from the villages of Tororo district, (Tb- *T. brucei*, Tc- *T. congolense* and Tv- *T. vivax*). Microscopy n = 28, PCR n = 124.

The results indicate that out of the 16 cattle found to be infected with trypanosomes by microscopic examination, 1 animal (6%), had *T. brucei*, 7 (44%), had *T. congolense* while 8 (50%) had *T. vivax* infections. The results also indicate that no animal had mixed infections of *T. brucei* /*T. congolense*, (Tb/Tc) or *T. brucei*/*T. vivax* (Tb/Tv) while 4 (26%) had mixed infections of both *T. congolense*/*T. vivax* (Tc/Tv) and 1 animal (4%) had mixed infection with all the three species (Tb/Tc/Tv). The results also indicate that out of the 161 cattle found to be infected with trypanosomes by PCR amplification, 51 animals (32%) had *T. brucei* infection, 66 (41%) had *T. congolense* while 43 (27%) had *T. vivax* infections. The result also show that 13 animals (8%), had mixed infections of *T. brucei*/*T. congolense* 11 (7%) had *T. brucei*/*T. vivax* while 5 (3%) had *T. congolense*/*T. vivax* and 5 (3%) had mixed infections of all species (Figure 29).

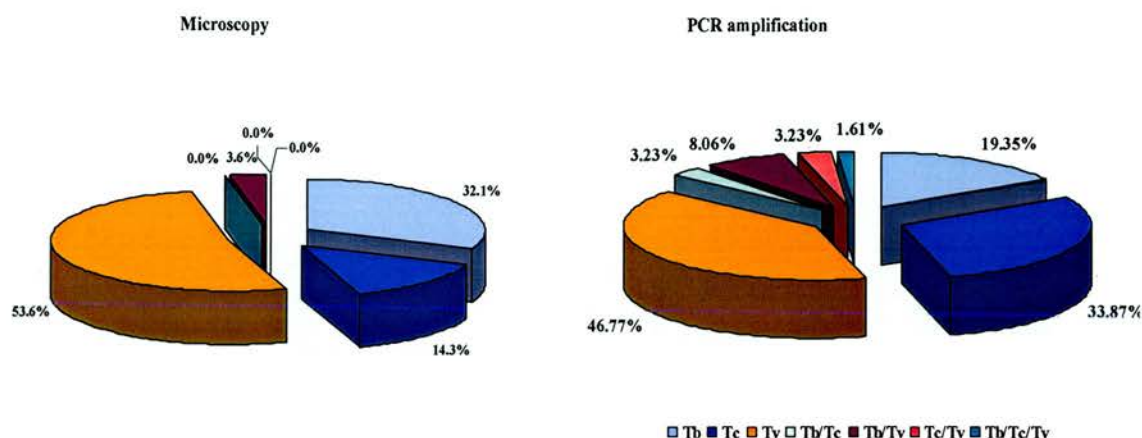


Figure 29: Proportion of animals with various trypanosome species from the villages of Busia district, (Tb- *T. brucei*, Tc- *T. congolense* and Tv- *T. vivax*) Microscopy n = 16, PCR n = 161

The proportion of animals detected with each of the three trypanosome species, from the villages of Tororo and Busia districts, by microscopy and PCR amplification is shown in Figure 30. The results indicate that out of the 44 cattle found to be infected with trypanosomes by microscopic examination 10 animals (22%) had *T. brucei*, 7 (18%), had *T. congolense* while 23 (52%) had *T. vivax* infections. The results also indicate that no animal had mixed infections of both *T. brucei* /*T. congolense*, (Tb/Tc) 1 (2%) had *T. brucei*/*T. vivax* (Tb/Tv), while 4 (9%) had *T. congolense*/*T. vivax* (Tc/Tv) and 1 (2%) had mixed infection with all the three species (Tb/Tc/Tv) infections. The results also indicate that out of the 285 cattle found to be infected with trypanosomes by PCR amplification, 74 animals (26%) had *T. brucei*, 108 (38%) had *T. congolense* while 103 (36%) had *T. vivax* infections. The result also showed that 23 animals (8%), had mixed infections of *T. brucei*/*T. congolense*, 20 (7%) had *T. brucei*/*T. vivax* while 9 (3%) had *T. congolense*/*T. vivax* and 9 animals (3%) had mixed infections with all the three species.

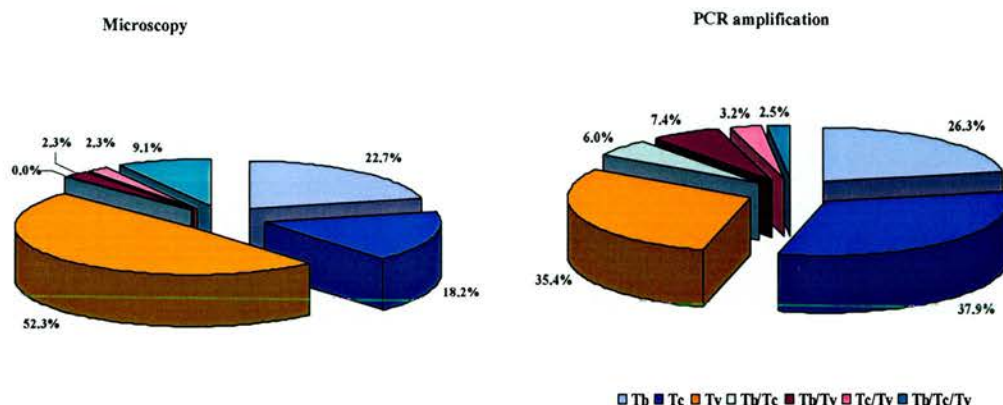


Figure 30: Proportion of various trypanosome species in cattle from the villages of Tororo and Busia district, (Tb- *T. brucei*, Tc- *T. congolense* and Tv- *T. vivax*) Microscopy n = 44, PCR n = 285

#### 4.3.3.3 The proportions of *T. congolense* types in cattle from Tororo and Busia villages

The proportions of various *T. congolense* types in cattle from the villages of Tororo and Busia districts as was determined from PCR amplification is shown in Figure 31. The results indicate that in Tororo, out of the 41 cattle that were found to be infected with *T. congolense* 17 animals had the Forest type representing 42%, 21 had Savannah type (51%) while 3 or 7% had Tsavo type as 1 animal or 2% had mixed infections of the Savannah and Forest types, Figure 31 (a).



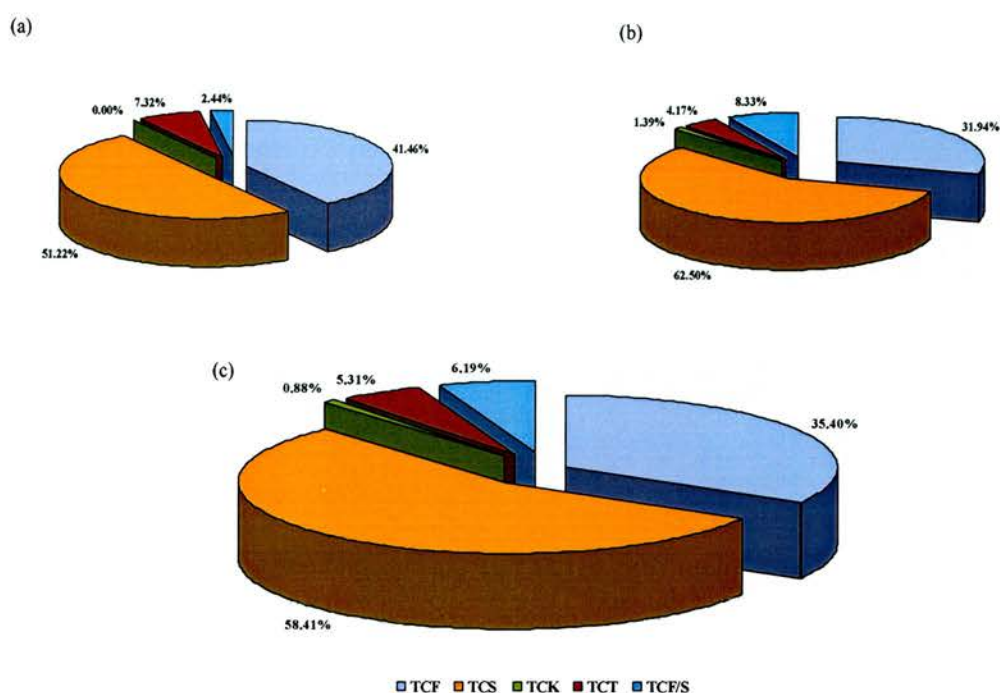


Figure 31: Proportions of *T. congolense* types in cattle from (a) Tororo, (b) Busia and (c) Both districts. TCF- Forest type, TCS- Savannah type, TCK- Kilifi type and TCT- Tsavo type.

The proportion of various *T. congolense* types in cattle from Busia villages is shown in Figure 31 (b). The results indicate that out of the 72 cattle infected with *T. congolense* 45 had Savannah type representing 63%, 23 had the Forest type (32%), 1 had the Kilifi type (1%) and 3 had the Tsavo type (4%) as 6 animals or 8% had mixed infections of the Savannah and Forest types. The proportion of various *T. congolense* types in cattle from the villages of both districts, as determined by PCR amplification, is shown in Figure 31 (c). The results indicate that out of the 113 cattle infected with *T. congolense* 63 (57%), had the Savannah type, 42 (37%), had the Forest type 1 (~0.5%) had the Kilifi type and 6 (5.5%) had the Tsavo type as 6 animals (5%) had mixed infections of the Savannah and Forest types.



#### 4.3.4 The prevalence of tick-borne parasites in cattle from Tororo district

The prevalence of *Theileria* species in cattle from the four villages of Tororo, as determined by PCR amplification, is shown in Figure 32. The results indicate that in Bunghanji village the prevalence of *T. parva* was 12.5% the 95% exact binomial confidence limits (CL) were between 4.2 and 26.8), *T. mutans* was 0% and *T. velifera* was 0%. In Hitunga the prevalence of *T. parva* was 10%, (CL between 2.8 and 23.7), *T. mutans* 57.5% (CL between 40.9 and 73.0) while the prevalence of *T. velifera* was 27.5% (CL between 14.6 and 43.9). In Magoje the prevalence of *T. parva* was 5.6% (CL between 0.7 and 18.6), *T. mutans* was 52.6% (CL between 38.1 and 72.1) while the prevalence of *T. velifera* was 13.9% (CL between 4.7 and 29.5). In Ojilai the prevalence of *T. parva* was 19.5% (CL between 11.3 and 30.1), *T. mutans* was 57.7% (CL between 36.9 and 76.6) while *T. velifera* was 7.7% (CL between 0.9 and 25.1).

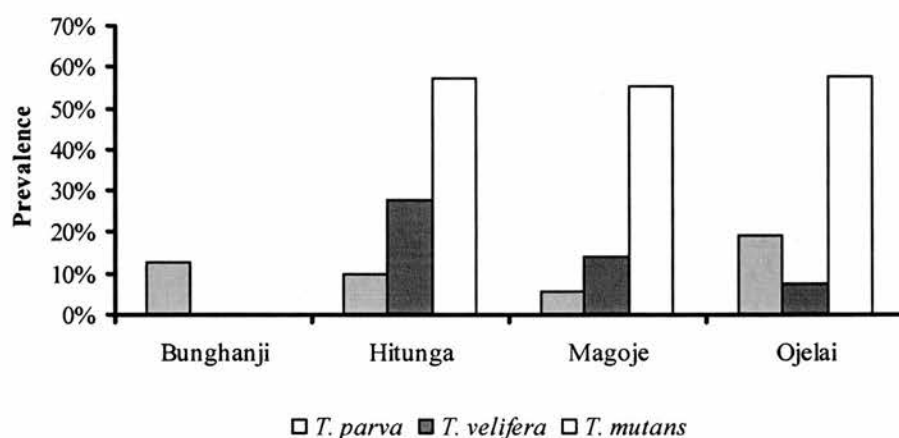


Figure 32: The prevalence of *Theileria* species in cattle from individual villages in Tororo as determined by molecular methods. The 95% exact binomial confidence limits are cited in the text.

The overall prevalence of various *Theileria* species in cattle from Tororo villages, as determined by PCR amplification, is shown in Figure 33. Out of a total of 319 cattle 212 were found to be infected with *Theileria* parasites, representing an overall prevalence of 66.5%. The prevalence of *T. parva* was 11.9% (confidence limits were 8.6 and 16.0), *T.*

*mutans* 42.3% (confidence limits 36.8 and 47.9) while *T. velifera* was 12.2% (confidence limits were 8.8 and 16.3).

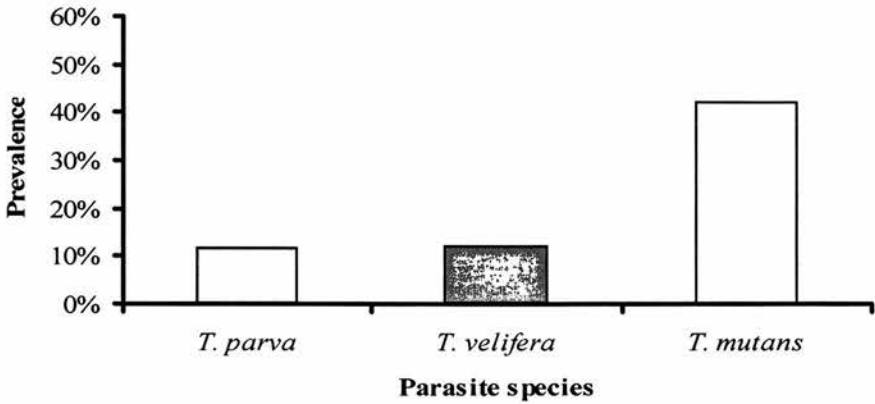


Figure 33: The prevalence of *Theileria* species in cattle from all the villages in Tororo as determined by molecular methods. The 95% exact binomial confidence limits are cited in the text.

The prevalence of tick-borne parasites in cattle from the four villages of Tororo district also determined by microscopic examination is shown in Figure 34. In Bunghanji the prevalence of *T. parva* was 58% (CL 46 and 68), while the prevalence of *Anaplasma marginale* was 48% (CL 36.2 and 59.0). In Hitunga the prevalence of *T. parva* was 49% (CL 38 and 61), *Babesia bigemina* was 1% CL 0.03 and 6.9) while *A. marginale* was 56% (CL 45.3 and 68.1). In Magoje the prevalence of *T. parva* was 61% (CL 49.7 and 72) and that of *A. marginale* was 63% (51 and 73). In Ojilai the prevalence of *T. parva* was 53% (CL 41 and 63.8) while that of *A. marginale* was 66% (CL 54.8 and 76.4).

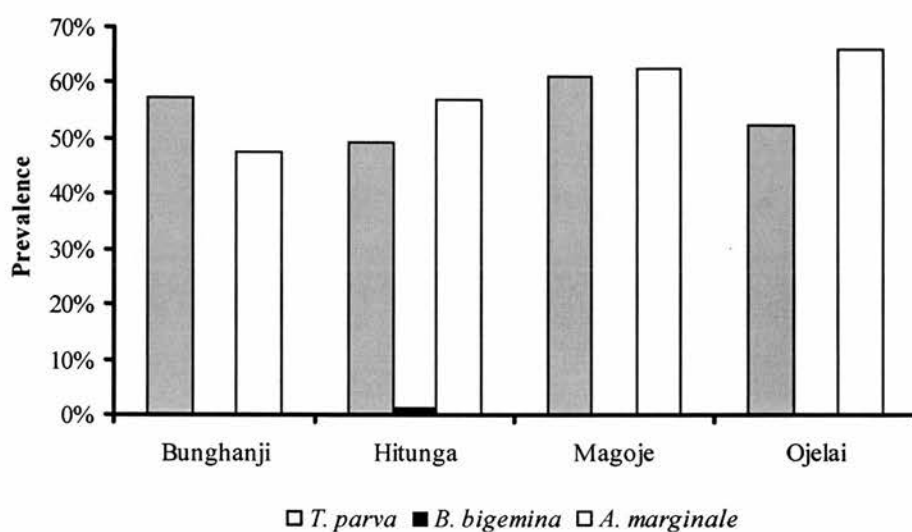


Figure 34: The prevalence of tick-borne parasites in cattle from the villages of Tororo district as determined by microscopy.

The overall prevalence of tick-borne parasites in cattle from Tororo villages as determined by microscopy is shown on Figure 35. The results indicate that out of a total of 363 animals detected to be positive with tick-transmitted parasites *T. parva* constituted 49% (CL 49.5 and 60.7), *A. marginale* was 51% (52.7 and 63.8) while *B. bigemina* was less than 1% (1).

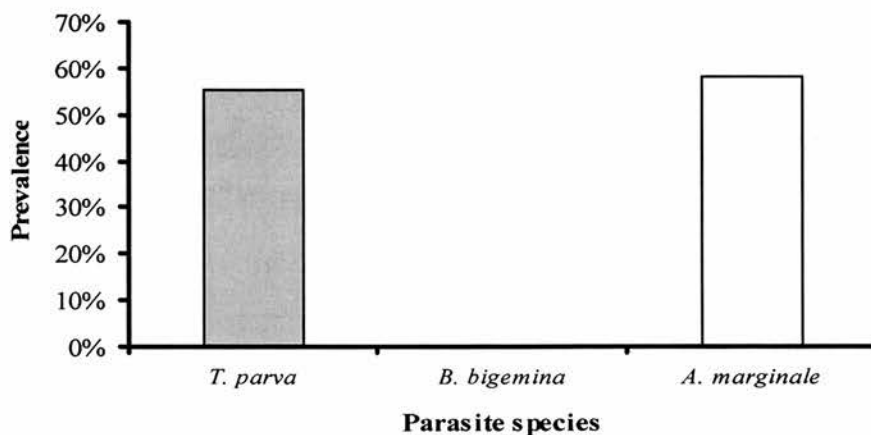


Figure 35: The prevalence of tick-borne parasites in cattle from Tororo district determined by microscopy.

The overall prevalence of *Theileria* species in cattle from Tororo and Busia as detected by microscopy is shown in Figure 36. The results indicate that the prevalence of *Theileria* species in cattle from Tororo district was 59% while in Busia it was 38%. This was statistically significant,  $\chi^2 = 27.7$ ,  $p < 0.0001$ .

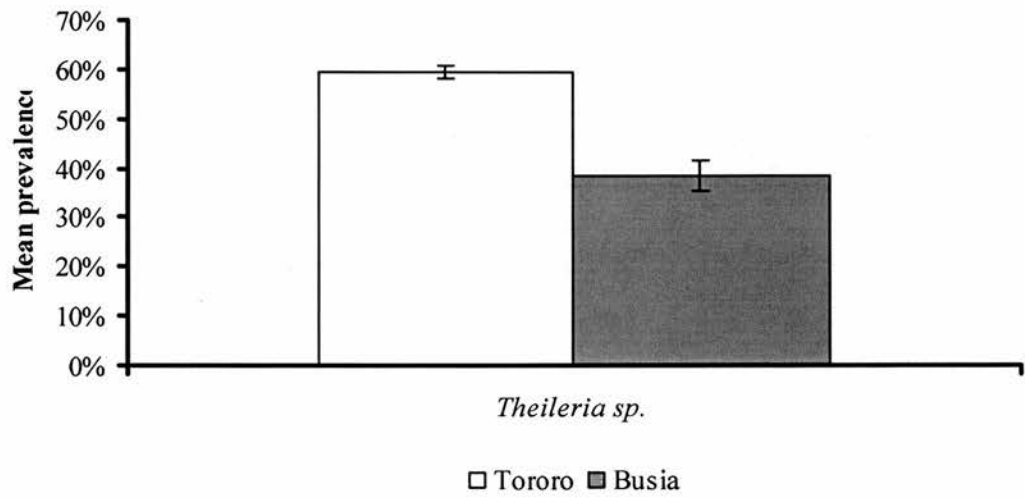


Figure 36: The mean prevalence of *Theileria* species in cattle from Tororo and Busia districts,  $n = 319$  in Tororo and  $320$  in Busia.

The overall prevalence of *Anaplasma* species in cattle from Tororo and Busia is shown in Figure 37. The results show that the prevalence of *Anaplasma* species as determined by microscopic examination was 56% while in Busia it was 33%. This was also highly significant,  $\chi^2 = 34$ ,  $p < 0.0001$ .

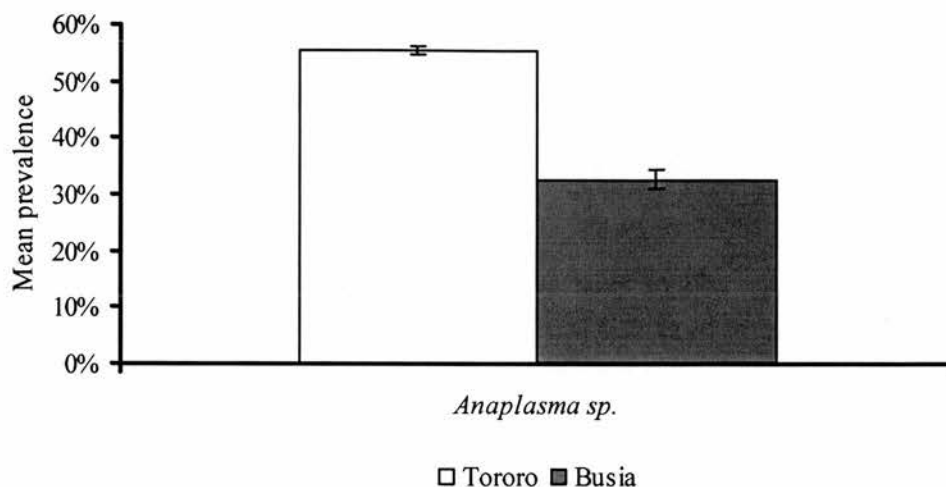


Figure 37: The mean prevalence of *Anaplasma* species in cattle from Tororo and Busia districts, n = 639.

## 4.4 DISCUSSION

### 4.4.1 Evaluation of the diagnostic performance of microscopy against PCR amplification on trypanosomes

Despite the fact that molecular methods have been shown to be highly sensitive and specific, (Masake, *et al.*, 1997; Desquesnes, 1997; de Almeida, *et al.*, 1997; Duvallet, *et al.*, 1999; Masake, *et al.*, 2003), their application in disease control and research programmes in the field has been limited to a handful of studies on cattle (Clausen, *et al.* 1998; Solano, *et al.* 1999; Mugittu, *et al.* 2001), and goats (de Almeida, *et al.*, 1997; Masake, *et al.*, 1997). This is largely due to the costs involved in performing molecular diagnostic methods and the need for more specialized equipment required for these methods. Microscopy has therefore prevailed in most field studies as a traditional method, despite the fact that it has been suspected for some time that it misses a large proportion of the infections present in livestock, due to its low sensitivity. For example, in one of the studies in which Paris *et al.* (1982) evaluated the various diagnostic methods for trypanosome species it was found that overall microscopy could only detect parasitaemia down to 100 parasites per ml. However, this was a carefully controlled and stringent examination where up to 200 microscopic fields were examined and is not normally possible to achieve with many animals being

screened under field conditions. The method is however relatively easy to carry out and since it has been in use for many years its cost is much lower compared to the molecular methods.

In the present study, a total of 636 cattle were examined for *T. vivax*, *T. congolense* and *T. brucei* by microscopy and PCR amplification as described in Chapter 3. The results showed that microscopy was able to detect 5 animals (4.9%) while PCR detected 102 animals (16%) as having *T. vivax* infection. Microscopy missed 97 cases (15.2%), which were detected by PCR amplification while it diagnosed 9 cases (1.4%) as *T. vivax* that were not detected by PCR amplification (Table 14) which could have been *T. brucei* cases misclassified by microscopy. The motility of *T. vivax*, its major distinctive diagnostic characteristic, reduces with time when blood is kept at room temperature (McOdimba, 1990), while the sizes of the two species overlap, thus making it difficult to distinguish them from *T. brucei* under the microscope, (Uilenberg, 1998). Only 5 out of 106 cases positively detected by PCR amplification were also detected by microscopy.

The probability of microscopic examination leading to the correct decision when the animal is infected with *T. vivax* (relative sensitivity) was found to be 0.05 (5%). The probability of microscopic examination leading to the correct decision when the animal is not infected with *T. vivax* (relative specificity) was 0.97 (97%) against PCR amplification (Table 18). The diagnostic predictive value showed that the probability of microscopy detecting positive *T. vivax* in cattle was 0.2 while the probability of detecting *T. vivax* negative cattle was 0.8. This is an indication that while reliability on microscopy detecting animals with *T. vivax* infection is unsatisfactory its reliability in detecting animals without infection is good. The kappa value was determined to be 0.02, suggesting that, based on PCR amplification as the gold standard, the reliability of microscopy in detecting *T. vivax* in cattle is not satisfactory, as it represents a poor agreement.

The results showed that microscopy detected 11 animals (1.7%) while PCR detected 106 animals (16.7%) as having *T. congolense* infections. Microscopy missed 104 cases (16%),



which were detected by PCR amplification while it misdiagnosed 18 cases (2.8%) as *T. congolense* that were not detected by PCR amplification. Only 2 cases out of 106 positively detected by PCR amplification were detected by microscopy (Table 15).

The probability of microscopic examination leading to the correct decision when the animal is infected with *T. congolense* (relative sensitivity) was found to be 0.019 (1.9%). The probability of microscopic examination leading to the correct decision when the animal is not infected with *T. congolense* (relative specificity) was 0.98 (98.3%) against PCR amplification. As with *T. vivax*, this is an indication that microscopy has poor sensitivity, possibly due to the fact that the parasitaemia of this trypanosome in cattle was lower than the microscopy threshold. However, the method had good specificity in detecting *T. congolense* in cattle (Table 17) suggesting that it is easy to identify the trypanosome when seen under the microscope.

The diagnostic predictive value showed that the probability of microscopy detecting positive *T. congolense* in cattle was 0.2. The probability of detecting *T. congolense* negative cattle was also 0.2, indicating that the reliability of microscopy in determining whether an animal has *T. congolense* infection or not is poor. The kappa value was determined to be 0.003. This implies a poor agreement, following the suggestions of Fleiss, (1981), where a kappa cut off equal to or lower than 0.4 represents poor agreement. Based on PCR amplification as the gold standard, the conclusion that can be drawn from these results is that the reliability of microscopy in detecting *T. congolense* in cattle is not satisfactory as it.

The results also showed that microscopy was able to detect 10 animals (1.2%) while PCR amplification detected 75 animals (11.8%) as having *T. brucei* infection. Microscopy missed 75 cases (11.8%), which were detected by PCR amplification while it misdiagnosed 10 cases (1.6%) as *T. brucei* that were not detected by PCR amplification. As has been mentioned with *T. vivax*, these cases that were detected by microscopy and not by PCR amplification could have been misclassified *T. vivax* infections. There were no cases

diagnosed by both microscopy and PCR amplification therefore the sensitivity, specificity and the diagnostic predictive values for microscopic detection of *T. brucei* in cattle could not be determined (Table 16). The overall performance of microscopy compared to PCR amplification in detecting any trypanosome species was found to be poor, kappa value = - 0.02 with a sensitivity of 4.9%, although the specificity was 94%, suggesting that microscopy is likely to give accurate results when the animals have no trypanosome infection, but would miss the positive cases.

The results obtained in this study are in agreement with the findings of Ngayo, *et al.*, (2005), where they found that out of the 402 animals sampled from a group of small ruminants in Busia Kenya, microscopy only detected 5 cases of trypanosomiasis of which only one was *T. congolense* infection while PCR amplification detected 21 cases of *T. congolense* infections. Masake, *et al.*, (1997), also found that whereas microscopy was able to reveal 42% cases of *T. vivax* infection, PCR revealed 75% in the same animals. In their study in Uganda, Clausen *et al.*, (1999), also found that microscopy showed the prevalence of trypanosomes to be 18.9% against 34.8% by PCR amplification and that out of these positive detections by microscopy *T. vivax* constituted 10.9% while PCR showed that they made up 20.6%.

As for *T. brucei*, the results in this study are in agreement with previous studies conducted by Clausen *et al.*, (1999), where they found that in a group of 486 cattle microscopy detected 26 animals infected with *T. brucei* against 48 detected by PCR amplification. Picozzi and others (2002) also found that microscopy detected 13 cases of *T. brucei* in cattle blood against 47 detected by PCR amplification using FTA filter cards. More recently, Ngayo, *et al.* (2005) found that microscopy could only detect 3 animals infected with *T. brucei* in small ruminants against 20 that were detected by PCR amplification.

Overall, the present results were in agreement with several studies that have compared the use of microscopy with that of PCR amplification in detecting *T. brucei* in goats (de Almeida, *et al.*, 1998) where it was shown that the overall positivity rate of PCR was

superior as it detected 97% positive compared to 74% with microscopy. In a study with cattle infected with *T. vivax*, the superiority of PCR against microscopy was again evident, where PCR amplification revealed 94% infections while microscopy showed only 63%; (Masake, *et al.*, 2002). In a study with *T. vivax* infections in goats, (de Almeida, *et al.*, 1997), it was shown that PCR amplification yielded twice as many positive cases as microscopy. Finally, it has been shown that PCR amplification detected 86% mouth part infections in tsetse flies compared to 51% by microscopic examination (Morlais, *et al.*, 2001).

Considering the number of animals that were detected in this study with *T. congolense*, *T. vivax* and *T. brucei* by PCR amplification but missed by microscopy, (an average of 14%) it is clear that the use of microscopy in diagnosing animal trypanosomiasis underestimates the real disease situation and is therefore unsuitable for quantifying the disease burden in cattle. The sensitivity of microscopy in detecting any trypanosome species was also low, 4.9%, although the specificity was found to be 93.7%. The kappa value was very low, -0.02, indicative of no agreement between the two methods. The infections detected by microscopy and missed by PCR amplification could be attributed to lack of specificity in classifying the species of trypanosomes detected by microscopy. In all the above mentioned previous studies however, no statistical analyses involving the sensitivity or specificity were done. The results obtained in this study have confirmed that the sensitivity of microscopy in detecting all the three trypanosomes species, in relation to PCR amplification is unsatisfactory. In conclusion, the analysis has resulted in important statistical comparisons that can be used in decision making with regards to the data collected using microscopy in detecting trypanosomes in animals for epidemiological studies.

#### **4.4.2 PCR amplification of *p104* gene of *T. parva* in cattle**

The detection of *Theileria parva* schizonts in cattle blood was achieved through PCR amplification of the *p104* gene, by using either the internal primers or both external and internal primers in a nested PCR. The sensitivity of PCR amplification with internal

primers was found to be 5% while the specificity was 89%. This is an indication that the internal primer PCR technique can be used to detect *T. parva* but not other the species of *Theileria*, but is not sensitive enough to detect low parasitaemia. The predictive value probability for detecting p104 positives was 0.18 and for detecting p104 negatives was 0.93. The Kappa value was 0.12 (Table 18) an indication that there was no agreement between the two methods. These results imply that the use of internal primers in PCR amplification of p104 gene in detection of *T. parva* is unsatisfactory when based on the nested PCR amplification method. The results are in agreement with the finding by Odongo (2004), where it was shown that the sensitivity of PCR using internal primers alone was very low compared to the nested PCR amplification.

#### 4.4.3 The prevalence of trypanosome species in cattle

The baseline prevalence of *T. brucei* in cattle from Tororo villages, as determined by PCR amplification, was found to be 7.5%, *T. congolense* 13% while *T. vivax* was 18.5% (Table 20). This was three times higher for *T. brucei*,  $\chi^2 = 6.17$ ,  $p < 0.05$ ; ten times for *T. congolense*  $\chi^2 = 30$ ,  $p < 0.05$  and four times for *T. vivax*  $\chi^2 = 28$ ,  $p < 0.05$ , than was determined by microscopy. The prevalence of *T. brucei* in cattle from Busia villages was 16%, *T. congolense* 21% while *T. vivax* was 13% (Table 21). This was fifty-three times higher for *T. brucei*,  $\chi^2 = 36$ ,  $p < 0.001$ ; ten times for *T. congolense*  $\chi^2 = 52$ ,  $p < 0.001$  and five times higher for *T. vivax*  $\chi^2 = 25$ ,  $p < 0.001$ . These results further confirm that PCR amplification detects more animals with trypanosome infections than microscopy and are in agreement with what had been found in a previous study by Clasuen *et al.*, (1998), where they showed that PCR amplification gave positive results in 34.8% of the blood samples tested as compared to 19% by m-AECT and 9.9% by HCT.

The prevalence of *T. brucei* and *T. congolense* was higher in cattle from Busia than in Tororo (*T. brucei* – 16% vs. 7.5%,  $\chi^2 = 22$ ,  $p < 0.0001$ ; *T. congolense* – 21% vs. 13%  $\chi^2 = 7.4$ ,  $p = 0.006$ ) while the prevalence of *T. vivax* was not higher in cattle from the villages of Tororo than in Busia,  $\chi^2 = 3.5$ ,  $p = 0.06$ , although the proportions were different, 18.5% vs. 13% as determined by PCR amplification. However, by microscopic examination the

prevalence of *T. brucei* was significantly higher in cattle from the villages of Tororo than in Busia, 2.8% vs. 0.3%,  $\chi^2 = 5$ ,  $p = 0.03$  while the prevalence of *T. vivax* and *T. congolense* was not different, 4.7% vs. 2.5%  $\chi^2 = 2.2$ ,  $p = 0.14$  and 2% vs. 1.3%  $\chi^2 = 0.1$ ,  $p = 0.75$  respectively.

When the data from the two districts were pooled together the prevalence of *T. brucei* and *T. congolense* was found to be 10 times while *T. vivax* was 5 times higher by PCR amplification than by microscopy (Table 22). The results obtained by PCR amplification were also higher than the 1.59%, 3.11% and 1.73% for *T. brucei*, *T. congolense* and *T. vivax* respectively, previously recorded in the area by microscopic examination of both thick and thin smears from a total of 47,240 animals (Ford, 1971).

In Tororo, a total of 124 cattle (39%) out of the 319 animals examined by PCR amplification from the analysis of the baseline samples, were found to be infected with trypanosomes. Out of these 19% were infected with *T. brucei*, 34% with *T. congolense* while 47% were *T. vivax* infections. Mixed infections of *T. brucei/T. congolense* were 3%, *T. brucei/T. vivax* were 8%, *T. congolense/T. vivax* were 3% while mixed infections with all the three species were 1.6% as shown in Figure 28. In Busia, a total of 320 cattle were examined by PCR of which 161 (50%) were found to be infected with trypanosomes. Out of these infections 32% were *T. brucei*, 41% were *T. congolense* while 27% were *T. vivax* infections. This was consistent with previous studies where *T. brucei* was predominant in areas endemic for sleeping sickness (Magona, *et al.*, 1999). Mixed infections of *T. brucei/T. congolense* were 8%, *T. brucei/T. vivax* were 7%, *T. congolense/T. vivax* were 3% while mixed infections with all the three species were 3% (Figure 30). The overall prevalence of trypanosomiasis in the two districts was found to be 44.6%, (exact binomial CI = 40.7 – 48.6,  $n = 639$ ). This was higher than the 25% prevalence previously mentioned for Uganda in general (Okuna, *et al.*, 1996) and the 7.1% observed by Magona and Mayende (2002), on farms in Tororo and Soroti districts of SE Uganda in particular using microscopy.

These results indicate that *T. vivax* was predominant in Tororo while *T. congolense* was predominant in Busia. However, there were twice as many cattle infected with *T. brucei* in Busia as were in Tororo (32% vs. 19%,  $\chi^2 = 13.67$ ,  $p < 0.0001$ ). These observations were in agreement with what was previously found in a study conducted in the same area by microscopy (Magona *et al.*, 2003) which showed that *T. brucei* was the predominant species. This was also in agreement with what had been found by PCR amplification by Clausen *et al.*, (1998) in Kampala, where they showed that 76.2% of the positive samples were *T. brucei*, 20.6% positive for *T. vivax* and 3.2% positive for mixed (*T. brucei/T. vivax*) infections, although *T. vivax* was predominant in Tororo.

In conclusion, the results in this study have further confirmed that the prevalence of all trypanosome species is much higher when determined by PCR amplification than microscopy. The results have also shown that the prevalence of trypanosomiasis in Tororo and Busia districts when determined by PCR amplification is much higher than previously documented in the area. The results have further confirmed that some of the animals infected with *T. brucei* s.l. may actually be carrying human infective *T.b. rhodesiense*. Underestimation of the prevalence of trypanosomiasis can therefore lead to serious consequences, not just for control of disease in livestock but also for human health.

The proportions of various *T. congolense* types in cattle from the villages of Tororo and Busia districts as determined by PCR amplification showed that 17 animals had *T. congolense* Forest type representing 42%, while Savannah type made up 51% and 7% were Tsavo type (Figure 31a). Only 1 animal or 2% had mixed infections of the Savannah and Forest types. The proportion of various *T. congolense* types in cattle from Busia villages were 63% Savannah type; 32% Forest type; while 1% were Kilifi type; Tsavo type made up 4% (Figure 31b). The proportion of various *T. congolense* types in cattle from the villages of both districts, as determined by PCR amplification were 57% Savannah type; 37% Forest type; while Kilifi type made up less 1% were and 6% were the Tsavo type. The results indicate that the Savannah type was more predominant in both districts followed by the Forest type. In a previous study, it had been found that *T. congolense* Savannah type



was more pathogenic than the other types (Sidibe, *et al.*, 2002). This would not be consistent with its higher prevalence in SE Uganda as it would have resulted in higher loss in cattle.

#### **4.4.4 The prevalence of TBD in cattle from Tororo and Busia**

The data obtained by p104 nested PCR amplification and reverse line blot (RLB) in this study showed that in cattle from Tororo *T. mutans* was the predominant species, with an overall prevalence of 42.2% (CI = 36.8 – 48.0) while the prevalence of *T. velifera* at 12.2%, (CI = 8.8 – 16.0) and *T. parva* 11.9% (CI = 8.6 – 16.0) were similar (Figure 33). This is in conformity with previous findings in an endemic region of central Uganda, (Oura *et al.*, 2004), where the prevalence of *T. mutans* was found to be higher than *T. velifera* and *T. parva*. In another study in Tanga and Iringa regions of Tanzania, (Ogden, *et al.*, 2003), it was observed that the high proportion of infection with *Theileria* parasites was due to higher prevalence of *T. mutans*. In Kenya (Watt, *et al.*, 1998) piroplasms were seen in cattle blood but *T. parva* could not be demonstrated by PCR amplification, leading to the conclusion that other *Theileria* species rather than *T. parva* were being detected by microscopy. Combining the prevalence of *Theileria* species determined by molecular methods shows that the overall prevalence was 66.3% which was higher than the 48% previously reported in this region, (Anon, 1996). The prevalence of *Theileria* species was significantly higher in Tororo, 59% than in Busia, 38%,  $P < 0.0001$ . The prevalence of *Anaplasma* was also significantly higher in Tororo 56% compared with Busia villages 33%,  $p < 0.0001$  but once again it was not possible to determine what species were present.

It is important to note that RLB failed to detect any *Anaplasma*, *Babesia* or the *Rickettsial* species in the same samples despite the fact that microscopy detected high prevalence of *Anaplasma* at 58%. The seroprevalence of *Anaplasma* in a few selected animals in this study was also high at 74%, while *Babesia* was 67% (Magona, thesis, 2004 Glasgow University). Generally, the prevalence of tick-borne parasites as screened by microscopy was higher in cattle from Tororo villages than in Busia (Figures 36 and 37). None of the animals screened in Tororo and Busia were positive for *Cowdria* and only 3 were positive

for *Babesia*. However, it was not possible to determine which species they were infected with, since the cattle were only screened by microscopy.

While molecular methods such as PCR amplification and reverse line blot (RLB) assays provided the basis for specific species identification, in this study they lacked the ability to detect *Anaplasma* or *Babesia* parasites. In particular, although RLB provided information on the prevalence of other *Thieleria* species such as *T. mutans* and *T. velifera*, its sensitivity in detecting *T. parva* and other tick-borne parasites was questionable since it did not detect any animal positive. This was despite the fact that microscopy was able to detect high prevalence of *Anaplasma* (up to 51% in cattle from Tororo district). While the results are consistent with the observations by Odongo (2004) that the sensitivity of RLB is too low for detecting *Anaplasma* and *Babesia*, this could also have been due to the fact that the samples were not stored in the manner that would have preserved these parasites. The storage of blood, which involved the use of glycerol in phosphate buffered saline with glucose (PSG), was meant to preserve trypanosomes. There is therefore need for further investigation to determine the applicability of this method in screening field samples. It is also important to note that the RLB method was found to be far more labour intensive and too costly for the screening of large number of samples and therefore is unsuitable as a diagnostic technique applicable for field evaluation of disease prevalence.

The examination of Giemsa stained blood smears could only distinguish parasites at species level. With the exception of *Anaplasma* and *Babesia* detection, the sensitivity and specificity of microscopy has been found to be generally low compared to the PCR amplification method. However, microscopy still remains the only method that is capable of providing both morphological and locomotive characteristics as well as quantitative data on the parasitaemia of micro-organisms in infected animals, although attempts are now being made through modeling techniques to quantify trypanosomes in the blood of animals following PCR amplification, (Andrew Cox, personal communications).

In conclusion, the results in this study have clearly shown that molecular techniques provide higher and more accurate prevalence estimations than microscopy, especially with

trypanosomiasis. The molecular methods also have the advantage of allowing for specific species identification.

The results in this study have clearly shown that the prevalence of various vector borne parasites infecting cattle in this area is much higher than previously estimated. In particular, the prevalence of trypanosomiasis in this area of SE Uganda had been highly underestimated in the previous studies carried out by microscopy (Okuna, *et al.*, 1996; Magona and Mayende (2002). However, the use of microscopy still provided the best option for estimating the prevalence of tick-borne parasites other than *Theileria* species. Furthermore, many epidemiological studies still require morphological characterization of disease pathogens that can only be achieved by microscopy. It is therefore reasonable to suggest that the use of molecular techniques be applied quantitatively while microscopy should be used to complement them in providing qualitative evaluation of disease pathogens in livestock.

## CHAPTER FIVE

### 5. MONTHLY PREVALENCE OF TRYPANOSOMES IN CATTLE

#### 5.1 INTRODUCTION

In this Chapter the prevalence of the three trypanosome species and *T. congolense* types as well as *T. b. rhodesiense* in cattle was monitored monthly. The prevalence of *T. brucei* was followed over a period of 16 months in Tororo and 13 months in Busia cattle. The prevalence of *T. congolense* and *T. vivax* was followed for a period of six months in both districts. The monthly incidence of all the three species of trypanosomes was also determined in cattle for six months. A number of longitudinal studies on prevalence and incidence of trypanosomes have been done in cattle. For example, Rowlands, *et al.* (1993) in their study of epidemiology of bovine trypanosomiasis in the Ghibe valley in southwest Ethiopia, determined the monthly prevalence and incidence in different animal age groups as well as the prevalence with seasonality in a total of 480 animals. Agyemang, *et al.* (1992) in their study on the interaction between physiological status in N'Dama cows and trypanosome infections and its effect on health and productivity of cattle in the Gambia, collected data on incidence of trypanosome infections and degree of anaemia on 420 animals for three years. Tewelde, *et al.* (2004) while studying the application of field methods to assess isometamidium resistance of trypanosomes in 300 cattle in western Ethiopia, monitored the animals for trypanosomes for 84 days. In all these studies the animals were screened by microscopy.

In the present study, the screening of animals was done using PCR amplification with primers specific for *T. brucei*, *T. congolense* and *T. vivax* as described in Chapter 3. Since it was not known which type of *T. congolense* was prevalent in the area four sets of primers targeting the four types of *T. congolense*, namely, *T. congolense* Forest type, *T. congolense* Kilifi type, *T. congolense* Savannah type and *T. congolense* Tsavo type, were used. This was done in order to ensure that the overall prevalence of all *T. congolense* types in cattle was not missed by increasing both the sensitivity and specificity of PCR amplification Clausen, *et al.*, (1999) used primers targeting Savannah type and no *T. congolense* was

detected. Both prevalence and incidence of the three trypanosome species were determined on cattle under prophylactic drug intervention with isometamidium chloride and compared with those without treatment.

The prevalence of *T. b. rhodesiense* was determined by PCR screening of a total of 104 *T. brucei* sl positive samples following PCR amplification with TBR primers. A total of 23 samples were from the baseline, 7 from September, 2 from October, 9 from November, 38 from December and 29 were from January. All the samples were screened with *SRA* gene targeting primers as described in Chapter 3.

### **5.1.1 Objectives of the study in this Chapter**

The objective of this study was to determine the monthly prevalence of the three species of trypanosomes, *T. brucei*, *T. congolense* and *T. vivax*, in cattle from the villages of Tororo and Busia districts and to determine the incidence of the three species of trypanosomes in cattle. In particular, the study aimed at comparing the prevalence of the trypanosome species in cattle under rural farming management system with and without prophylactic drug intervention. The Chapter also aimed to determine the effect of treatment of cattle with isometamidium chloride on haemoglobin concentration. The aim of this analysis was to assess the impact of treatment on anaemia in trypanosomiasis.

## **5.2. MATERIALS AND METHODS**

### **5.2.1 Monthly prevalence of trypanosomes in cattle**

Monthly prevalence of *T. brucei* in cattle from the villages of Tororo was determined over a period of 16 months between July 2001 and September 2002 while the prevalence from the villages of Busia district was determined over a period of 13 months between October 2001 and September 2002. The monthly prevalence of *T. congolense* and *T. vivax* was done over a period of 6 months, between July 2001 and February 2002 in Tororo and October 2001 and April 2002 in Busia. The monthly incidence of each trypanosome species in cattle was determined over a period of six months in both districts. The total number of cattle screened during each month in the study is listed in Chapter 2, Table 4.



Individual trypanosome species prevalence was determined from the results of PCR amplification by the formula:

$$\text{Prevalence} = (p/n) * 100$$

Where p is the number of positive observations or animals and n is the total number of animals screened.

The 95% exact binomial confidence intervals (CI), for the determined prevalence at each time point prevalence was computed using R-statistical software version 2.1.1. In order to compare the proportions between treated and untreated animals prevalence was calculated for each time point for each trypanosome species by Pearson's Chi-square with Yates continuity correction following the formula:

$$\text{Chi-square} = \Sigma ((O - E)^2) / E$$

$$\text{Where } E = (Gt * O) / T$$

Where O is the observed values (positives and negatives), E is the expected values, Gt is the group total and T is the total number of the animals examined.

Where the data set was too small and the expected values for positive results were below 5, Chi-squared test can be unreliable and Fisher's Exact Test was used. Both analyses were used to test for significance of differences in prevalence between the isometamidium chloride - treated and untreated groups of animals using the R-statistical software version 2.1.1. Since sampling of animals was started at different time points, (Tororo in July while Busia in October), the data from each district was considered separately to determine whether there would be any seasonality effect. The data was then pooled, by aligning the days from treatment time in both districts.



### **5.2.2 Effect of isometamidium chloride treatment on haemoglobin concentration**

Haemoglobin concentration in the blood of animals was monitored by using a HemoCue as described in Chapter 3. From the data, a group of animals that had no trypanosome infection prior to treatment and then subsequently had treatment were selected and categorized into treated and untreated groups, along with those that had infection but were given no treatment. The monthly mean haemoglobin concentration was computed from each group and compared between treated and untreated. Since the data did not conform to normal distribution a two sample t - test for significance difference was used

## **5.3 RESULTS**

### **5.3.1 Monthly prevalence of *T. brucei* in cattle from the villages of Tororo and Busia districts**

The prevalence of *T. brucei* in treated and untreated cattle from Tororo and Busia districts is shown in Figure 38. The results show that in Tororo the initial prevalence of 11.2% in the treated and 3.8% in the untreated cattle were significantly different,  $p = 0.02$ ,  $\chi^2 = 5.23$ . The results showed no significant difference in the subsequent months up to the fifth month after treatment. However, in month six, the results showed a significant difference in prevalence between treated (54.8%) and untreated cattle (12.7%),  $p < 0.0001$ ,  $\chi^2 = 49$ . In months 8 and 9 after treatment, the prevalence was significantly lower in the treated cattle than the untreated ones, 1.7% and 1.8%, vs. 8% and 9.5%,  $p < 0.05$ ,  $\chi^2 < 5$  in both months. The results also show that in month 13 there was a significant difference in prevalence between treated, 25.8% and untreated cattle, 9.0%,  $p = 0.004$ ,  $\chi^2 = 8.17$ , Figure 38 (a).

In Busia the initial prevalence of *T. brucei* in the treatment and non treatment villages was not different, 13.8% vs. 10.0%,  $p > 0.1$ ,  $\chi^2 = 0.8$ . However, in months 2, 3, 4, and 8 the prevalence was significantly lower in the treated cattle than the untreated cattle, 3.6%, 2.7%, 0% and 0%, vs. 13.9%, 6.7%, 9.6% and 11.6% ( $p < 0.01$ ,  $\chi^2 = 8.1, 5.4, 10.5$  and  $6.8$ ) respectively, in all the months,. However, in months 6 and 10, the prevalence in the treated cattle was significantly higher than the untreated ones, 25.5% vs. 9.5% and 44.4% vs.

17.2%, ( $p < 0.005$ ,  $\chi^2 = 8$  and 21) respectively, in both months. Subsequently the prevalence was significantly lower in the treated cattle than the untreated ones, 0% in the following four months against 14.8%, 16.2%, 13.9% and 12.5%, ( $p < 0.05$ ,  $\chi^2 = 6.2, 7.1, 5.6, 4.2$ ), in all the four months Figure 38 (b).

When the data from the two districts was combined the results showed that the initial prevalence of treatment cattle was significantly higher than the no treatment ones, 12.5% vs. 6.9%,  $p = 0.02$ ,  $\chi^2 = 5.5$ . In months 2 and 3, the prevalence was significantly lower in the treated cattle than in the untreated ones, 2.1% vs. 8.9% and 1.0% vs. 4.9%, ( $p < 0.01$ ,  $\chi^2 = 11.2$  and 6.3), in both months. However, in month six the prevalence was significantly higher in the treated cattle than in the untreated ones, 41.9% vs. 11.2%,  $p < 0.0001$ ,  $\chi^2 = 54.7$ . In months 8, 9, 12 and 14, the prevalence was significantly lower in the treated cattle than in the untreated ones, 1.1% vs. 9.5%, 3.1% vs. 10.4%, 1.4% vs. 7.9% and 0% vs. 5.2%, ( $p < 0.01$ ,  $\chi^2 = 12, 6, 5.6$  and 5.9), in all the months, Figure 38 (c).

The prevalence of *T. brucei* in individual villages of Tororo and Busia districts is shown in Figure 39. In the villages of Tororo, the initial prevalence determined in the month of July was 6.3% in Bunghaji, 3.8% in Ojilai, 8.6% in Hitunga and 13.8% in Magoje. In months 3 and 4 following treatment the prevalence remained below 5% in all the villages. However, in the month of November, the prevalence rose to 18.2% in Bunghaji but dropped to 0% the following month. The prevalence rose to 10% in Ojilai, 23.1% in Hitunga and 1.4% in Magoje in the month of November. In the month of December the prevalence rose to 18.2% in Ojilai, 42.79% in Hitunga and 9.4% in Magoje. The prevalence remained at 0% in Bunghaji and dropped to 12.7% in Ojilai but continued to rise to 74.7% in Hitunga and 30.0% in Magoje in the month of January, Figure 39 (a).

In the villages of Busia, the initial prevalence determined in the month of October was 7.5% in Buyimini, 12.5% in Sitengo, 13.8% in Kubo and Magoje. In the following month prevalence dropped to 0% in Buyimini and Kubo but remained at 12.8% and 5.3% in Sitengo and Nanjeho villages. The prevalence rose again to 7% and 20% in Buyimini and

Sitengo and to 7.4% in Nanjeho but remained at 0% in Kubo in the month of December. The prevalence remained below 2% in Buyimini, Kubo and Nanjeho in the months of January, February and March but rose to 12.3%, 16.9% and 22.0% in Sitengo during the same period. However, in the month of April, the 6<sup>th</sup> month, the prevalence rose to 2% in Buyimini, 16.7% in Sitengo, 38.3% in Kubo and 8.7% in Nanjeho, Figure 39 (b).

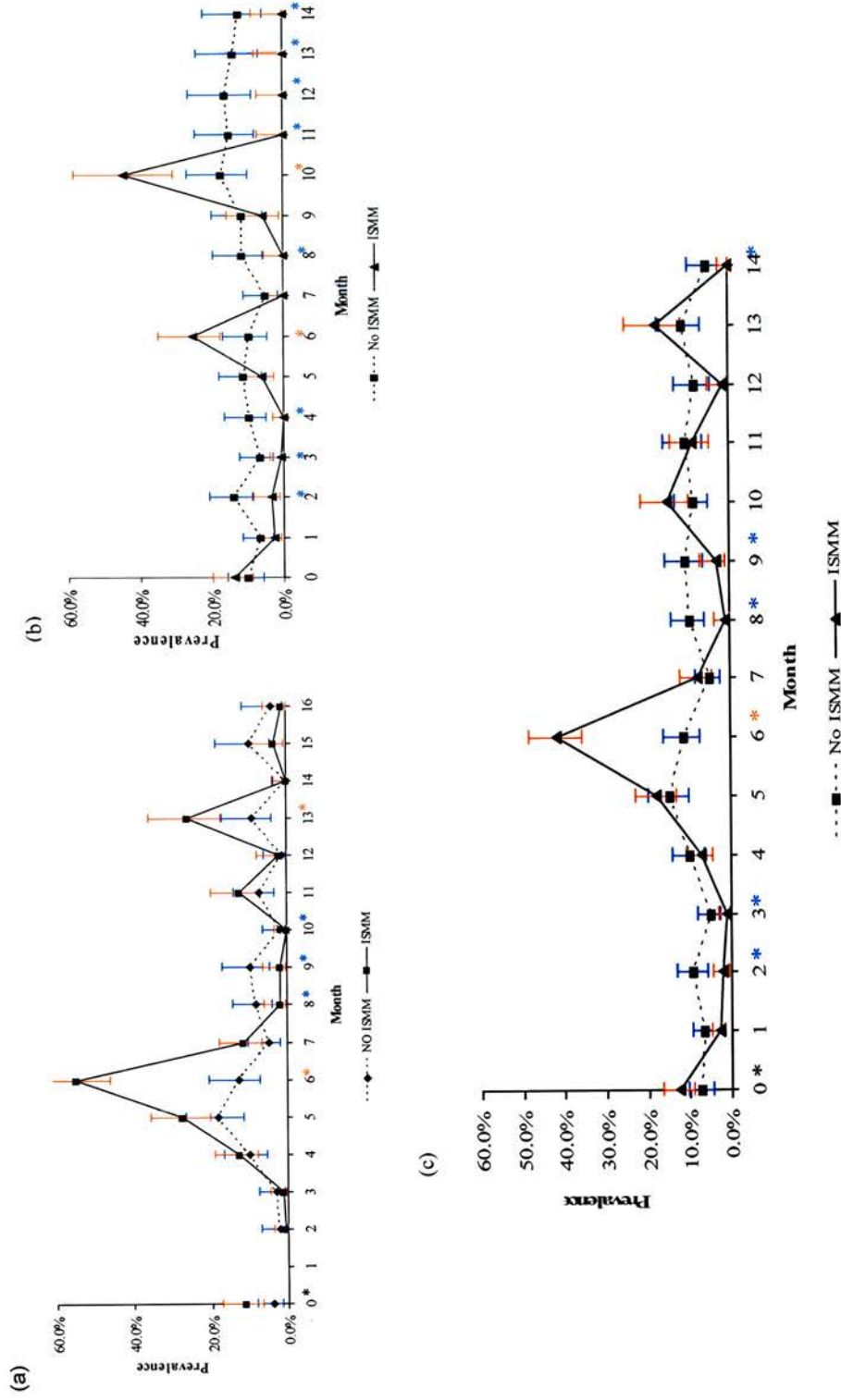


Figure 38: The Prevalence of *T. brucei* in cattle from (a) Tororo, (b) Busia and (c) both districts determined by PCR. The orange asterisks indicate the months when treatment had negative effect while the blue asterisks indicate the months when treatment had positive effect over no treatment.

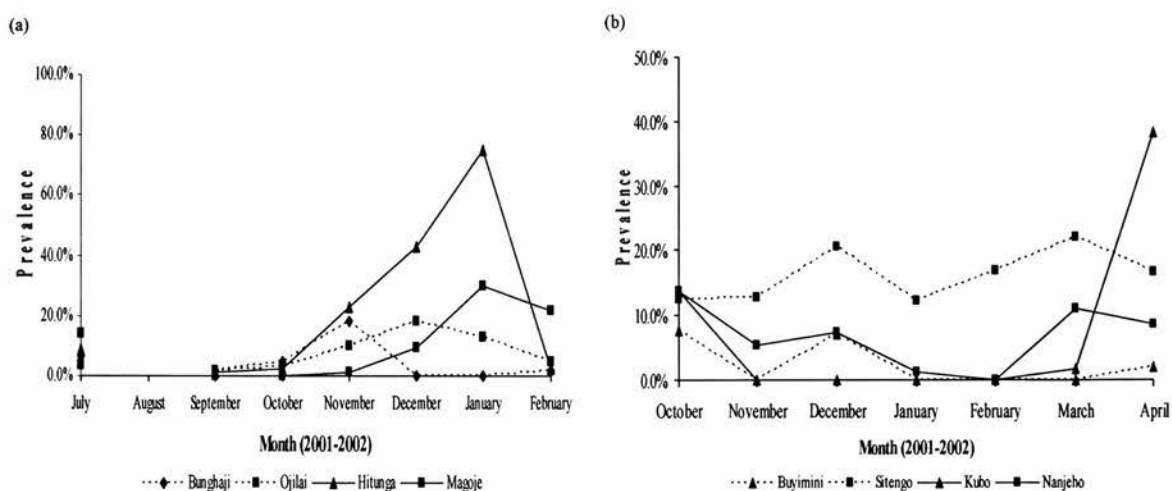


Figure 39: The Prevalence of *T. brucei* in cattle from the individual villages of (a) Tororo and (b) Busia Districts. The treatment villages are Hitunga (HT), Magoje (MA), Kubo (KU) and Nanjeho (NN) and the no treatment villages are Bung'haji (BH), Ojilal (OJ), Buyimini (BY) and Sitengo (ST).

### 5.3.1.1 The prevalence of *T.b. rhodesiense* in cattle

A total of 104 samples previously found positive with *T. brucei* (TBR) primers, were randomly selected, 23 from July samples, 7 from September, 2 from October, 9 from November, 38 from December while 29 were from January. The results show that out of the 20 samples from the baseline that successfully re-amplified with *T. brucei* targeting primers and 5 of them amplified with *SRA* primers. However, no samples from September, October, November and December re-amplified with *T. brucei* targeting primers, 50 from the baseline and 54 were from six months post treatment samples. Out of the 29 samples from January that were re-screened for *T. brucei*, 17 re-amplified while 4 of them amplified with *SRA* primers, Table 22.

Table 22: The prevalence of *T.b. rhodesiense* in cattle from Tororo villages

Month	No. of samples re-screened	<i>T. brucei</i> positive	SRA positive	SRA:T.b ratio
July	23	20	5 (25%)	1:4
September	7	0	0	-
October	2	0	0	-
November	9	0	0	-
December	38	0	0	-
January	29	17	4 (23.5%)	1:4

### 5.3.2 Monthly prevalence of *T. congolense* in cattle from the villages of Tororo and Busia districts.

The prevalence of *T. congolense* in treated and untreated cattle from Tororo and Busia districts is shown in Figure 40. The results show that in Tororo the initial prevalence in the treatment group of cattle was significantly higher than in the no treatment animals, 18.6% vs. 6.3%,  $p = 0.002$ ,  $\chi^2 = 9.8$ . The results showed no significant difference in the subsequent months up to the fourth month when the prevalence in the treated cattle was significantly lower than in the untreated animals, 1.3% vs. 26.2%,  $p < 0.0001$ ,  $\chi^2 = 37.9$ , Figure 40 (a).

In Busia the the initial prevalence in the treatment group of cattle and non treatment group was not different, 21.9% vs. 19.4%,  $p > 0.1$ . However, in the month 2, the prevalence was lower in the treated cattle than in the untreated ones, 0.7% vs. 7.6%,  $p = 0.01$   $\chi^2 = 6.7$ , before rising the following month to 9.4% in the treated cattle against 2.2% in the untreated group,  $p = 0.03$ ,  $\chi^2 = 5$ . The prevalence rose much higher in the month 4, in the treated than untreated cattle, 27.6% vs. 6.1%,  $p < 0.0001$ ,  $\chi^2 = 17$ , but dropped to 0.9% treated cattle compared to 6.8%, in the untreated animals,  $p = 0.04$ ,  $\chi^2 = 4$  in month 5, Figure 40 (b).

When the data from the two districts was combined the results showed that the initial prevalence of treatment cattle was significantly higher than the no treatment ones, 20.2% vs. 12.9%,  $p = 0.02$ ,  $\chi^2 = 5.7$ . In months 2 and 5, the prevalence was significantly lower in



the treated cattle than in the untreated ones, 1.1% vs. 4.5% and 0.4% vs. 4.4%,  $p < 0.03$ ,  $\chi^2 = 4.8$  and  $7.1$  respectively, Figure 40 (c).

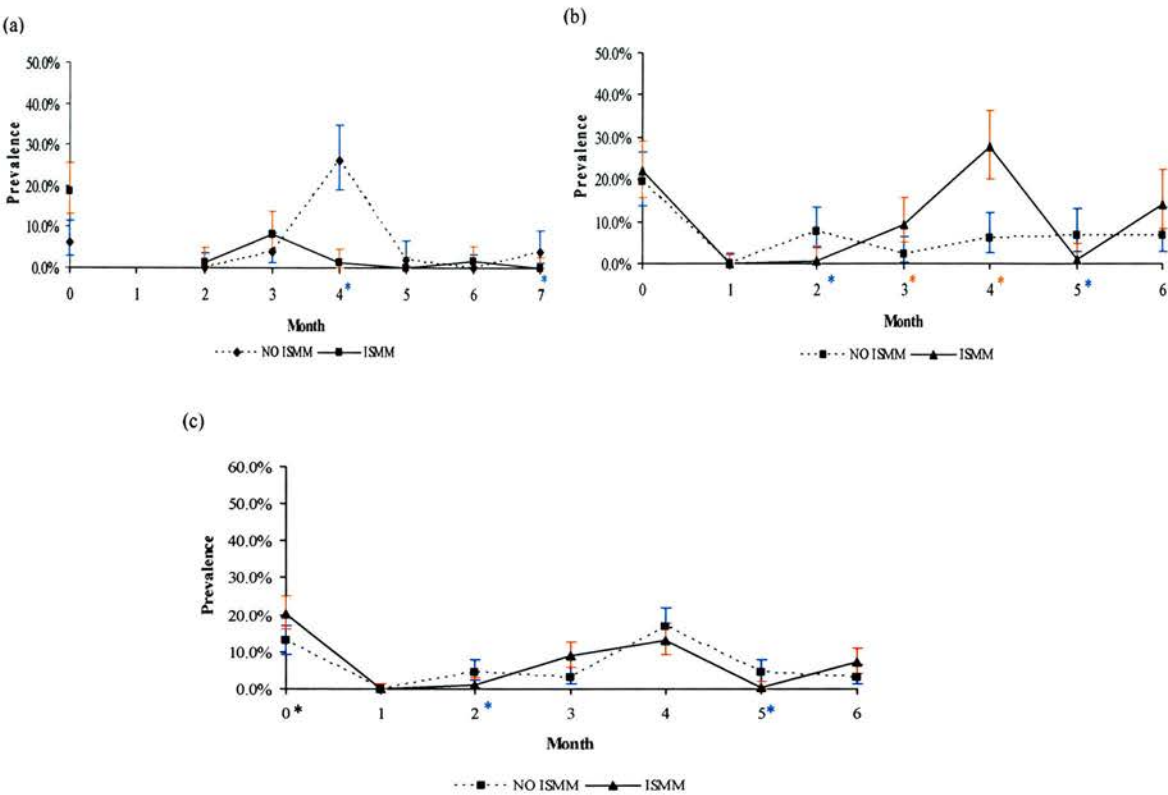


Figure 40: The Prevalence of *T. congolense* in cattle from (a) Tororo, (b) Busia and (c) both districts as determined by PCR. The orange asteriks indicate the months when treatment had negative effect while the blue asteriks indicate the months when treatment had positive effect over no treatment.

The prevalence of *T. congolense* in individual villages of Tororo and Busia districts is shown in Figure 41. In the villages of Tororo, the initial prevalence determined in the month of July was 3.8% in Bunghaji, 8.9% in Ojilai, 17.3% in Hitunga and 20% in Magoje. In the months of September and October, following treatment the prevalence dropped below 5% in all the villages. However, in the month of October, the prevalence rose to 1.5% in Bunghaji, 6.1% in Ojilai, 15.0% in Hitunga and 1.3% in Magoje. In the month of November the prevalence rose to 38.2% in Bunghaji, 17.3% in Ojilai, 2.6% in Hitunga and

0% in Magoje. Subsequently, the prevalence remained at 0% and less than 3% in Ojilai, Magoje and Hitunga but rose to 3.8% in December and 7.5% in February in Bunghaji, Figure 41 (a).

In the villages of Busia, the initial prevalence determined in the month of October was 8.8% in Buyimini, 30.0% in Sitengo, 18.8% in Kubo and 25.0% in Nanjeho. In the following month prevalence dropped to 0% in all the villages. In the month of December the prevalence rose again to 7.0% and 8.2% in Buyimini and Sitengo but only to 1.5% in Nanjeho and 0% in Kubo. In the month of January the prevalence rose to 17.1% in Nanjeho and 4.1% in Sitengo but remained at 0% in the other villages. In the month February the prevalence was 10.2% in Buyimini, 3.1% in Sitengo, 1.7% in Kubo and 30.0% in Nanjeho, Figure 41 (b).

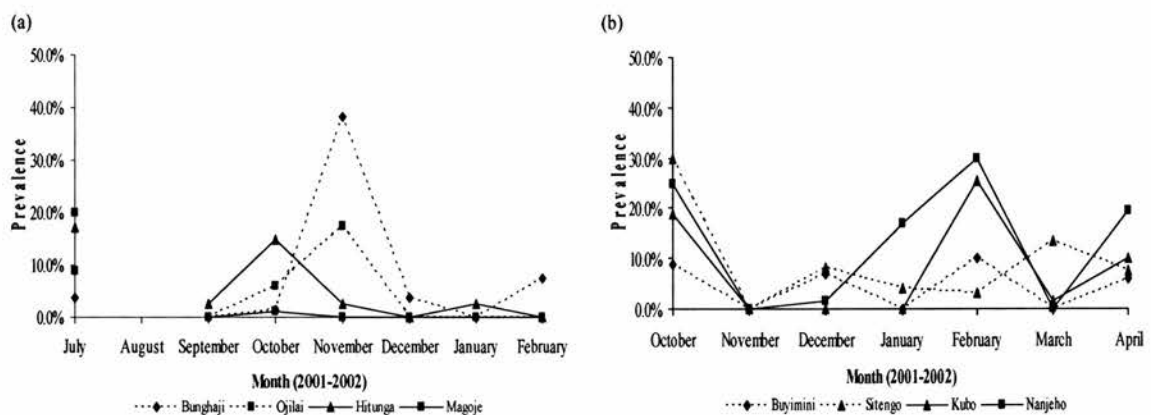


Figure 41: The Prevalence of *T. brucei* in cattle from the individual villages of (a) Tororo and (b) Busia Districts. The treatment villages are Hitunga (HT), Magoje (MA), Kubo (KU) and Nanjeho (NN) and the no treatment villages are Bunghaji (BH), Ojilai (OJ), Buyimini (BY) and Sitengo (ST).

### 5.3.3 Monthly prevalence of *T. vivax* in cattle from the villages of Tororo and Busia districts

The prevalence of *T. vivax* in treated and untreated cattle from Tororo and Busia districts is shown in Figure 42. The results show that in Tororo the initial prevalence in the treatment group of cattle was significantly higher than in the no treatment animals, 23.6% vs. 13.3%,  $p = 0.01$ ,  $\chi^2 = 6.1$ . The results showed that there was no significant difference in the subsequent months up to month 4 when the prevalence in the treated cattle was lower than in the untreated animals, 0.7% vs. 3.1%,  $p < 0.001$ ,  $\chi^2 = 13.2$ . Subsequently, there was no significant difference in prevalence between the two groups of cattle for six months, Figure 42 (a).

In Busia the initial prevalence in the treatment was lower than in the non treatment groups of cattle, 6.3% vs. 20.6%,  $p < 0.001$ ,  $\chi^2 = 12.9$ . This difference persisted in the following month where the prevalence in the treated cattle was 0.7% vs. 5.2%  $p = 0.04$ ,  $\chi^2 = 4$ , in the treated animals. Subsequently, there was no difference in prevalence in the two groups up to month 5, when the prevalence remained at 1.8% in the treated cattle but rose to 12.7% in untreated animals, ( $p = 0.006$ ,  $\chi^2 = 7$ ) (Figure 42b).

When the data from the two districts was combined the results showed that the initial prevalence of treatment cattle was not different from the no treatment ones, 15.0% vs. 17.0%,  $p = 0.5$ . However, in the following month the prevalence was lower in the treated cattle than the untreated, 0.3% vs. 2.8,  $p = 0.04$ ,  $\chi^2 = 4.13$ . Subsequently there was no difference in the prevalence between the two groups of animals (Figure 42c).

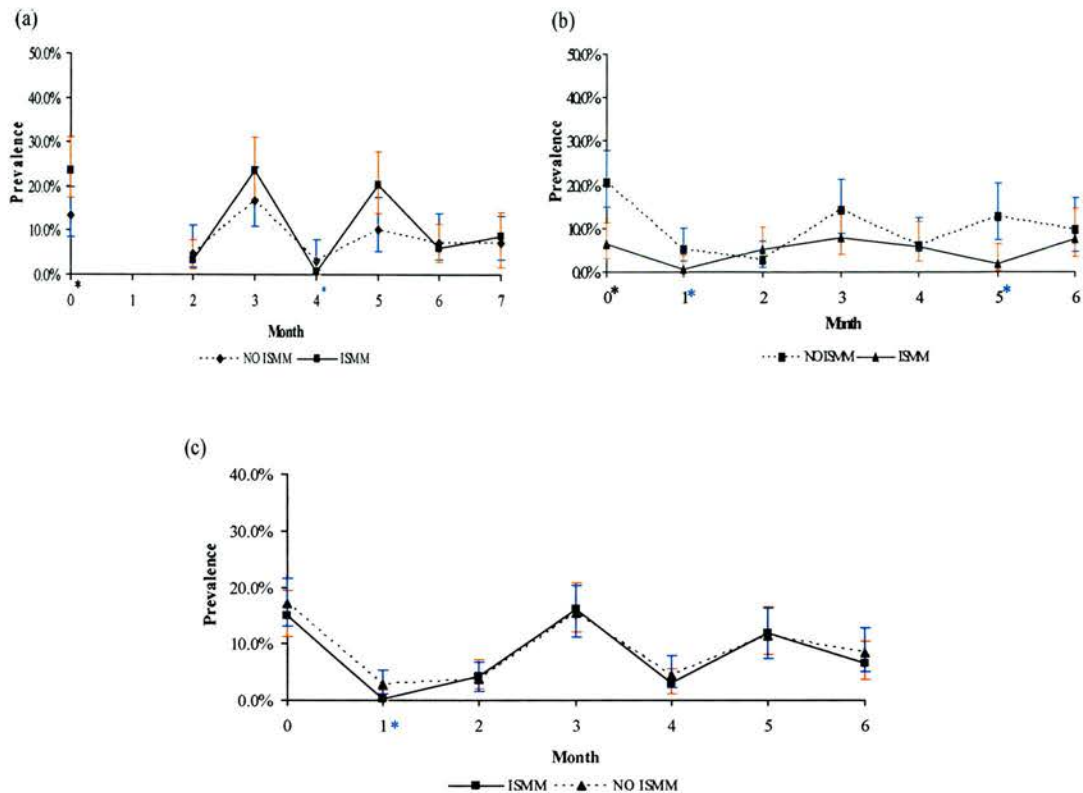


Figure 42: The Prevalence of *T. vivax* in cattle from (a) Tororo, (b) Busia and (c) both districts as determined by PCR. The blue asteriks indicate the months when treatment had positive effect over no treatment.

The prevalence of *T. vivax* in individual villages of Tororo and Busia districts is shown in Figure 43. In the villages of Tororo, the results show that the initial prevalence was 6.3% in Bunghaji, 20.3% in Ojilai, 11.1% in Hitunga and 36.3% in Magoje. In September the prevalence was 8.2% in Bunghaji, but dropped to 1.9% in Ojilai, 6.6% in Hitunga and 0% in Magoje. However, the prevalence rose to 25.8% in Bunghaji, 7.6% in Ojilai, 40.0% in Hitunga and 6.4% in Magoje in the month of October. In the month of November the prevalence dropped to below 4% in all the villages but rose to 5.8% in Bunghaji, 13.8% in Ojilai, 32.0% in Hitunga and 6.3% in Magoje (Figure 43a).

In the villages of Busia, the initial prevalence of *T. vivax* determined in the month of October was 15% in Buyimini, 26.3% in Sitengo, 5.0% in Kubo and 7.5% in Nanjeho. In the following month the prevalence dropped to 0% in Buyimini and Kubo, 10.3% in Sitengo and 1.3% in Nanjeho. In January the prevalence rose to 14.5% in Buyimini, 13.7% in Sitengo, 7.9% in Kubo and Nanjeho (Figure 43b).

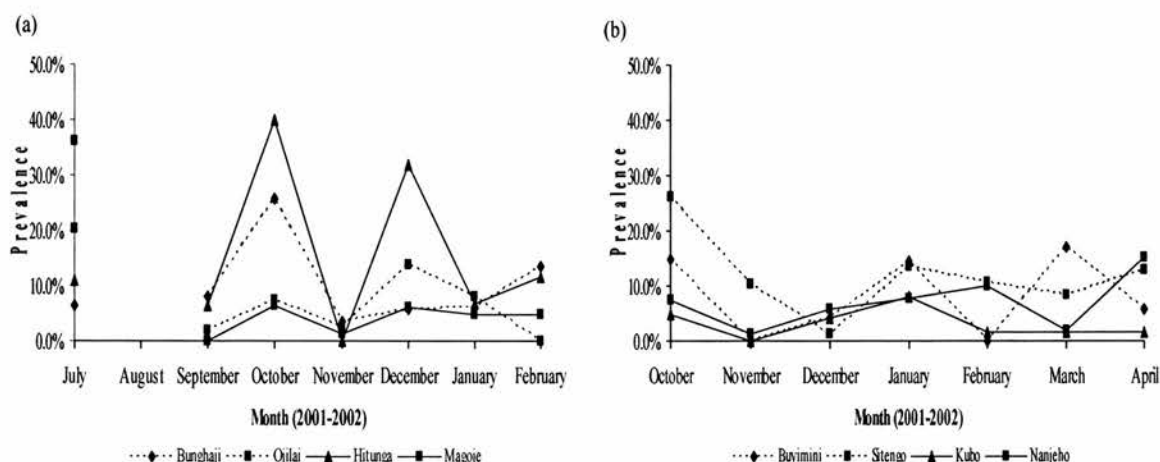


Figure 43: The prevalence of *T. vivax* in cattle from the individual villages of (a) Tororo and (b) Busia districts. The treatment villages are Hitunga (HT), Magoje (MA), Kubo (KU) and Nanjeho (NN) and the no treatment villages are Bunghaji (BH), Ojilai (OJ), Buyimini (BY) and Sitengo (ST).

### 5.3.4 The monthly prevalence of any trypanosome species in cattle from Tororo and Busia districts

The prevalence of any trypanosome species in the treated and untreated cattle from Tororo and Busia districts as determined by PCR amplification is shown in Figure 44. The results show that in Tororo the initial prevalence in the treatment group of cattle was significantly higher than in the no treatment animals, 42.2% vs. 21.5%,  $p = 0.0001$ ,  $\chi^2 = 14.8$ . The results showed that there was no significant difference in the subsequent months up to the fourth month when the prevalence in the treated cattle was lower than in the untreated animals, 14.7% vs. 34.6%,  $p < 0.0001$ ,  $\chi^2 = 15.5$ . However, in month six, the prevalence was

significantly higher in the treated cattle than the untreated ones, 56.3% vs. 16.4%,  $p < 0.0001$ ,  $\chi^2 = 43.3$  (Figure 44a).

In Busia there was no significant difference in initial prevalence in the treatment and no treatment groups, 36.3% vs. 41.9%,  $p = 0.76$ . However, in months one, two and five, following treatment, the prevalence was significantly lower in the treated cattle than the untreated ones, 3.4% vs. 11.1%, 8.8% vs. 22.9 and 8.8% vs. 26.3% respectively,  $p < 0.05$ ,  $\chi^2 = 5.9$ , 9.8 and 11.5. In month six, the prevalence in the treated cattle rose significantly higher than the in the untreated animals, 40.6% vs. 22.9%,  $p = 0.01$ ,  $\chi^2 = 6.6$  (Figure 44b).

When the data from the two districts was combined the results showed that the initial prevalence of treatment cattle was not different from the no treatment ones, 39.3% vs. 31.8%,  $p = 0.54$ . However, in the following two months the prevalence was lower in the treated cattle than the untreated, 1.7% vs. 6.0 and 6.3% vs. 15.9%,  $p < 0.03$ ,  $\chi^2 = 6$  and 11.9. Subsequently there was no significant difference in the prevalence between the two groups of animals up to month six, when the prevalence in the treated cattle rose significantly higher than the untreated animals, 49.4% vs. 19.5%,  $p < 0.0001$ ,  $\chi^2 = 45.5$  (Figure 44c).

The prevalence of any trypanosome species in cattle from the two districts as determined by microscopy is shown in Figure 45. The prevalence of any trypanosome species in Tororo district shows that there was significant difference in month three, 0.6% in the treated compared to 6.1% in the untreated animals, ( $p = 0.02$ ,  $\chi^2 = 5.4$ ). In month eight, the prevalence was 1.7% in the treated compared to 10.4% in the untreated animals, ( $p = 0.01$ ,  $\chi^2 = 6.3$ ) and in month fourteen, the prevalence was 0.9% in the treated and 7.9% in the untreated animals, ( $p = 0.03$ ,  $\chi^2 = 4.5$ ) (Figure 45a). The prevalence in Busia district shows significant difference in month four, 11.4% in the treated compared to 4.4% in the untreated animals, ( $p < 0.001$ ,  $\chi^2 = 13.6$ ) and month six, 10.4% in the treated vs. 4.8% in the untreated animals, ( $p = 0.02$ ,  $\chi^2 = 5.6$ ) (Figure 45b). Overall, the prevalence was not



different in treated and untreated cattle from both districts except in month one, 0% in treated and 4.6% in the untreated animals ( $p = 0.001$ ,  $\chi^2 = 12.2$ ), Figure 45 (c).

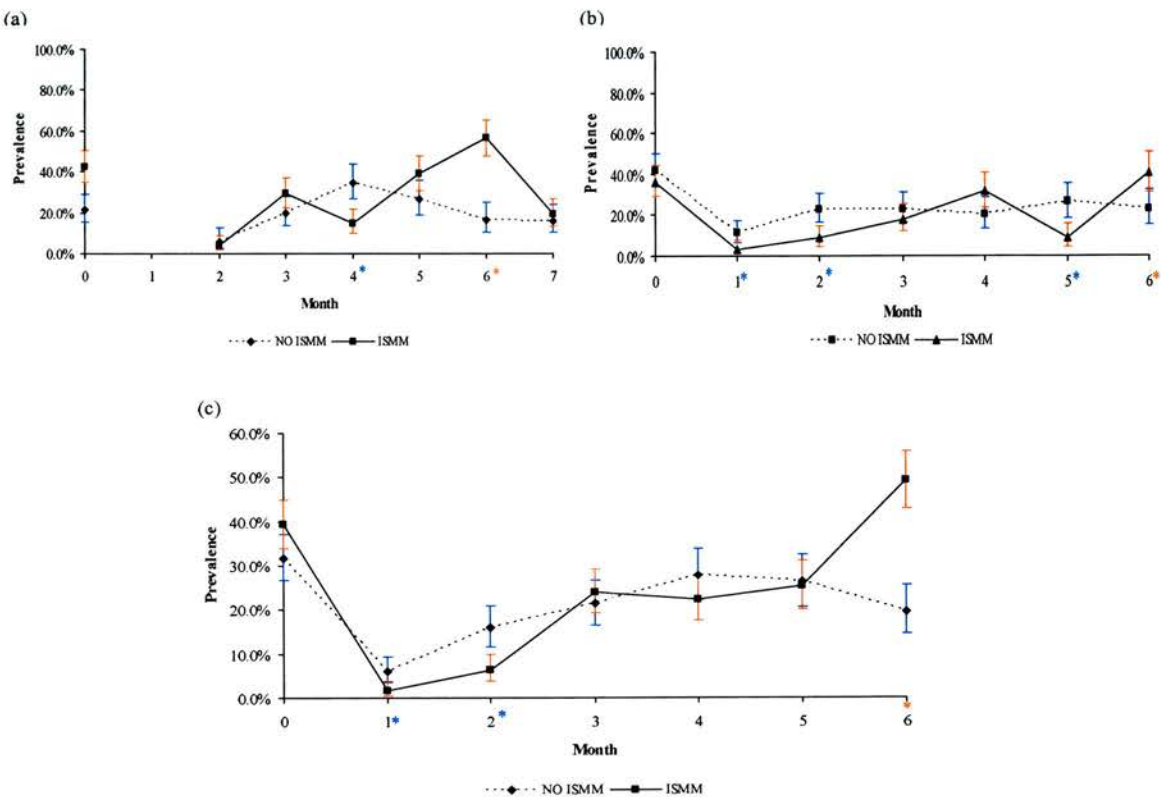


Figure 44: The prevalence of any trypanosome species in cattle from (a) Tororo, (b) Busia and (c) both districts determined by PCR amplification. The orange asteriks indicate the months when treatment had a negative effect while blue asteriks indicate the months when treatment had positive effect over no treatment.

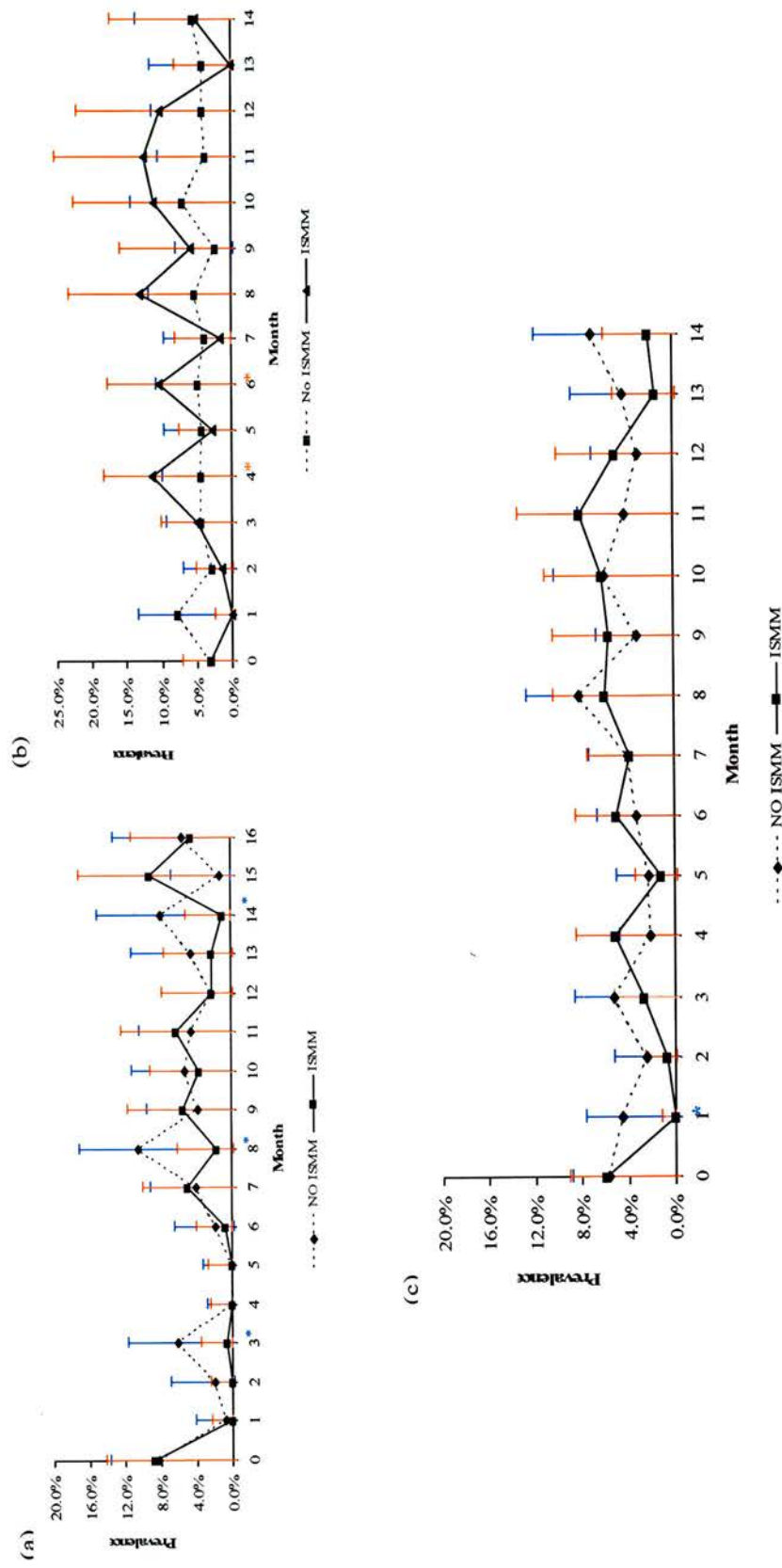


Figure 45: The prevalence of any trypanosome species in cattle from (a) Tororo, (b) Busia and (c) both districts determined by microscopy. The orange asteriks indicate the months when treatment had a negative effect while blue asteriks indicate the months when treatment had positive effect over no treatment.

The prevalence of any trypanosome species in cattle from the individual villages of Tororo and Busia districts as determined by PCR amplification is shown in Figure 46. In the villages of Tororo, the initial prevalence determined in the month of July was 13.9% in Bunghaji, 29.1% in Ojilai, 35.8% in Hitunga and 48.8% in Magoje. The prevalence in month of September was 10.2% in Bunghaji, 1.9% in Ojilai, 7.9% in Hitunga and 0% in Magoje. The prevalence continued to rise, reaching 52% in Bunghaji in November, 41.4% in Ojilai in December, 76.0% and 31.7% in Hitunga and Magoje in January, Figure 46 (a).

In the villages of Busia, the initial prevalence determined in the month of October was 26.3% in Buyimini, 57.5% in Sitengo, 32.5% in Kubo and 40% in Nanjeho. In the following month the prevalence dropped to 0% in Buyimini, 21.8% in Sitengo, 0% in Kubo and 6.7% in Nanjeho. Subsequently the prevalence rose to maximum peaks of 16.9% in Buyimini in the months of December and March, 35.6% in Sitengo in the month of March, 41.7% in Kubo in the month of April and 39.1% in Nanjeho in the month of April, Figure 46 (b).

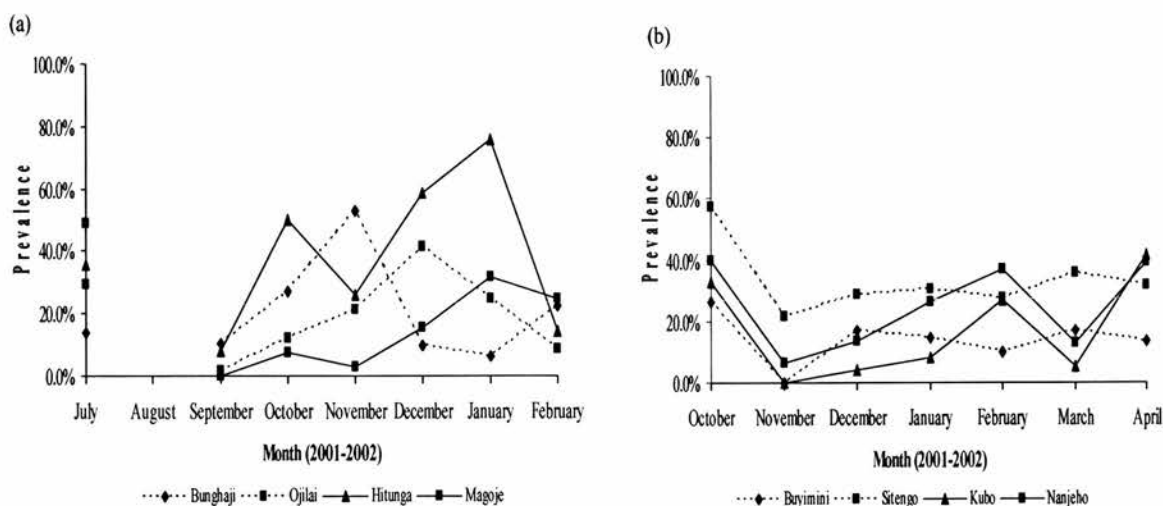


Figure 46: The prevalence of any trypanosome species in cattle from the individual villages of (a) Tororo and (b) Busia districts. The treatment villages are Hitunga, Magoje, Kubo and Nanjeho while the no treatment villages are Bunghaji, Ojilai, Buyimini and Sitengo.

The effect of isometamidium chloride treatment of cattle against the three trypanosome species in both districts, as determined by PCR amplification and microscopy, is summarized in Table 23(a) and (b). The results show that in Tororo, treatment had little effect on the prevalence of *T. brucei* in cattle. In Busia, the results show that treatment had some positive protective effect on cattle against infection with *T. brucei*. The results show that in month six, treatment had negative effect in protecting cattle against infection in both districts. The results show that treatment had a protective effect on *T. congolense* and *T. vivax* in Tororo on month 4, but had a negative effect on *T. congolense* infection in Busia district. The results however do not show any positive protective effect on any species of trypanosomes in both districts under microscopic examination.

Table23 (a): Summary of the effect of isometamidium treatment of cattle against trypanosome species determined by PCR amplification. (0 = no effect, + = protective effect, - = negative effect).

	Tororo				Busia				Combined districts			
Month	Tb	Tc	Tv	All sp	Tb	Tc	Tv	All sp.	Tb	Tc	Tv	All sp.
1	ND	ND	ND	ND	0	0	+	+	0	0	+	+
2	0	0	0	0	+	+	0	+	+	+	0	+
3	0	0	0	0	+	-	0	0	+	0	0	0
4	0	+	+	+	+	-	0	0	0	0	0	0
5	0	0	0	0	0	+	+	+	0	+	0	0
6	-	0	0	-	-	0	0	-	-	0	0	-
7	0				0				0			
8	+				+				+			
9	+				0				+			
10	+				-				-			
11	0				+				0			
12	0				+				+			
13	-				+				0			
14	0				+				+			
15	0				ND				0			
16	0				ND				0			

Table 23(b): Summary of the effect of isometamidium chloride treatment of cattle against trypanosome species determined by microscopy. (0 = no effect, + = protective effect, - = negative effect).

Month	Tb	Tc	Tv	All sp
1	0	+	+	+
2	0	0	+	0
3	0	0	0	0
4	0	-	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0
12	0	0	0	0
13	0	0	0	0
14	0	0	0	0

#### 5.4.1 Monthly incidence of *T. brucei* infections in cattle from Tororo and Busia districts

The incidence of *T. brucei* in cattle from Tororo villages is shown in Table 24 (a). The incidence was highest in the months of November, (31), December, (56) and January, (79). Of the 31 new cases in November, 12 were in the untreated villages, 19 were in the treated villages. Of the 56 new cases in December, 19 were in the untreated villages, 26 were in the treated villages while 11 were recurring after previously being detected. Of the 79 new cases in January, 3 were in the untreated villages, 42 were in the treated villages and 34 were recurring after previously being detected. The incidence was lowest in the months of August 2001, (0), September, (2), October, (5), May, (2) and July, (4).

The incidence of *T. brucei* in cattle from Busia villages is shown in Table 24 (b). The incidence was highest in the months of October, (37), April, (34) and August, (86). Of the 37 new cases in October, 15 were in the untreated villages, 22 were in the treated villages. Of the 34 new cases in April, 3 were in the untreated villages, 28 were in the treated villages while 3 were recurring after previously being detected. Of the 86 new cases in August, 29 were in the untreated villages, 54 were in the treated villages and 3 were

recurring after previously being detected. The incidence was lowest in the months of January 2002, (1), May, (0), September, (6) and November, (2).



Table 24(a): The monthly incidence of *T. brucei* in cattle from Tororo district, (bold figures in parenthesis are percentages).

Months	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov
	<b>0</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>	<b>13</b>	<b>14</b>	<b>15</b>	<b>16</b>
Untreated	24(15)	0	1(0.4)	3(1.1)	12(8)	19(12)	3(2)	7(4)	10(6)	5(3)	2(1)	7(4)	1(0.4)	15(9)	0	13(8)	12(8)
Treated	0	0	1(0.4)	2(0.7)	19(12)	26(16)	42(26)	12(8)	2(1)	0	0	17(11)	1(0.4)	25(16)	0	2(0.7)	2(0.8)
Recurring	0	0	0	0	0	11(2)	34(11)	4(1)	1(0.4)	2(0.8)	0	0	2(0.8)	10(3.1)	0	2(0.7)	3(1.2)

Table 24 (b): The monthly incidence of *T. brucei* in cattle from Busia district, (bold figures in parenthesis are percentages).

Months	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov
	<b>0</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>	<b>13</b>
Untreated	37(23)	4(3)	7(4)	0	4(3)	6(4)	3(2)	0	9(6)	4(3)	29(18)	6(4)	10(6)	2(0.8)
Treated	0	4(3)	5(3)	0	0	8(5)	28(18)	0	1(0.3)	2(0.7)	54(34)	0	0	0
Recurring	0	1(0.3)	0	1(0.4)	0	0	3(2)	0	0	2(1)	3(2)	0	2(1)	0

#### 5.4.2 Monthly incidence of *T. congolense* infections in cattle from Tororo and Busia districts

The incidence of *T. congolense* in cattle from Tororo villages is shown in Table 25 (a). The incidence was highest in the months of July, (40), October, (18) and November, (29). Of the 40 new cases in July, 10 were in the untreated villages, 30 were in the treated villages. Of the 18 new cases in October, 5 were in the untreated villages, 8 were in the treated villages while 5 were recurring after previously being detected. Of the 29 new cases in November, 27 were in the untreated villages, while 2 were recurring after previously being detected. The incidence was lowest in the months of August 2001, (0), September, (2), December, (0), January, (2) and February, (2).

Table 25(a): The monthly incidence of *T. congolense* in cattle from Tororo district, (bold figures in parenthesis are percentages).

Month	<u>Jul</u> <u>0</u>	<u>Aug</u> <u>1</u>	<u>Sept</u> <u>2</u>	<u>Oct</u> <u>3</u>	<u>Nov</u> <u>4</u>	<u>Dec</u> <u>5</u>	<u>Jan</u> <u>6</u>	<u>Feb</u> <u>7</u>
Untreated	40(25)	0	0	5(3)	27(17)	0	0	2(1)
Treated	0	0	2(1)	8(5)	0	0	2(1)	0
Recurring	0	0	0	5(3)	2(1)	0	0	0

The incidence of *T. congolense* in cattle from Busia villages is shown in Table 25 (b). The incidence was highest in the months of October, (66), January, (19) and February, (43). Of the 66 new cases in October, 31 were in the untreated villages, 35 were in the treated villages. Of the 19 new cases in October, 3 were in the untreated villages, 13 were in the treated villages while 3 were recurring after previously being detected. Of the 43 new cases in February, 8 were in the untreated villages, 30 were in the treated villages while 5 were recurring after previously being detected. The incidence was lowest in the months of November 2001, (0) and March 2002, (6).

Table 25 (b): The monthly incidence of *T. congolense* in cattle from Busia district, (bold figures in parenthesis are percentages).

Month	<u>Oct</u> <u>0</u>	<u>Nov</u> <u>1</u>	<u>Dec</u> <u>2</u>	<u>Jan</u> <u>3</u>	<u>Feb</u> <u>4</u>	<u>Mar</u> <u>5</u>	<u>Apr</u> <u>6</u>	<u>May</u> <u>7</u>
Untreated	66(41)	0	11(7)	3(2)	8(5)	5(3)	6(4)	0
Treated	0	0	0	13(8)	30(19)	0	11(7)	8(5)
Recurring	0	0	0	3(2)	5(3)	1(0.6)	9(6)	6(4)

#### 5.4.3 Monthly incidence of *T. vivax* infections in cattle from Tororo and Busia districts

The incidence of *T. vivax* in cattle from Tororo villages is shown in Table 26 (a). The incidence was highest in the months of July, (59), October, (57), December, (38) and February, (21). Of the 59 new cases in July, 21 were in the untreated villages and 38 were in the treated villages. Of the 57 new cases in October, 19 were in the untreated villages, 32 were in the treated villages while 6 were recurring after previously being detected. Of the 38 new cases in December, 10 were in the untreated villages, 16 were in the treated villages while 12 were recurring after previously being detected. Of the 21 new cases in February, 10 were in the untreated villages, 6 were in the treated villages while 5 were recurring after previously being detected. The incidence was lowest in the months of August 2001, (0) and November 2001, (2).

Table 26 (a): The monthly incidence of *T. vivax* in cattle from Tororo district, (bold figures in parenthesis are percentages).

Month	<u>Jul</u> <u>0</u>	<u>Aug</u> <u>1</u>	<u>Sept</u> <u>2</u>	<u>Oct</u> <u>3</u>	<u>Nov</u> <u>4</u>	<u>Dec</u> <u>5</u>	<u>Jan</u> <u>6</u>	<u>Feb</u> <u>7</u>
Untreated	59(37)	0	5(3)	19(12)	1(0.6)	10(6)	6(4)	10(6)
Treated	0	0	5(3)	32(20)	1(0.6)	16(10)	3(2)	6(4)
Recurring	0	0	0	6(4)	0	12(8)	5(3)	5(3)

The incidence of *T. vivax* in cattle from Busia villages is shown in Table 26 (b). The incidence was highest in the months of October, (43), January, (26), April, (33) and May, (22). Of the 43 new cases in October, 33 were in the untreated villages and 10 were in the treated villages. Of the 26 new cases in January, 15 were in the untreated villages, 9 were in the treated villages while 2 were recurring after previously being detected. Of the 33 new cases in April, 20 were in the untreated villages, 12 were in the treated villages while 1 was recurring after previously being detected. Of the 22 new cases in May, 11 were in the untreated villages, 9 were in the treated villages while 2 were recurring after previously being detected. The incidence was lowest in the months of November 2001, (7), December, (10 and February 2002, (11).

Table 26 (b): The monthly incidence of *T. vivax* in cattle from Busia district, (bold figures in parenthesis are percentages).

Month	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
	0	1	2	3	4	5	6	7
Untreated	43(27)	5(3)	3(2)	15(9)	3(2)	13(8)	20(13)	11(7)
Treated	0	0	6(4)	9(6)	5(3)	2(1)	12(8)	9(6)
Recurring	0	2(1)	1(0.6)	2(1)	3(2)	0	1(0.6)	2(1)

## 5.5 Effect of isometamidium treatment of cattle on haemoglobin concentration

The results of the monthly mean haemoglobin concentration in treated and untreated cattle from Tororo are shown in Table 29. The results indicate that there was no significant differences in the montly mean haemoglobin concentration between treated and untreated animals for the first five months ( $p > 0.05$ ). However, in the sixth month, the mean haemoglobin concentration was higher in treated animals, 10.69g/dl compared to 10.03g/dl in the untreated animals,  $t = -2.08$ ,  $df = 154$  and  $p = 0.04$ .

Table 27: Mean monthly haemoglobin concentration (g/dl) in treated and untreated cattle under infection with trypanosomes in Tororo.

Month	Mean (Treated)	Mean (Untreated)	d.f.	p – value	t - value
0	9.96	9.84	249	0.6	-0.47
1	10.46	10.06	218	0.1	-1.58
2	10.68	10.44	188	0.3	-1.02
3	10.81	10.37	158	0.1	-1.67
4	10.54	10.45	163	0.7	-0.36
5	10.32	10.35	153	0.9	0.14
6	10.69	10.03	154	0.04	-2.08

The results of the monthly mean haemoglobin concentration in treated and untreated cattle from Busia are shown in Table 30. The results indicate that there was no significant differences in the montly mean haemoglobin concentration between treated and untreated animals for the first five months,  $p > 0.05$ . However, as in Tororo, in the sixth month, the mean haemoglobin concentration was lower in treated animals, 9.60g/dl compared to 10.08g/dl in the untreated animals,  $t = 1.94$ ,  $df = 185$  and  $p = 0.05$ .

Table 28: Mean monthly haemoglobin concentration (g/dl) in treated and untreated cattle under infection with trypanosomes in Busia.

Month	Mean (Treated)	Mean (Untreated)	d.f.	p – value	t - value
0	9.75	9.93	317	0.4	0.79
1	10.14	10.03	297	0.6	-0.51
2	10.67	10.43	278	0.2	-1.22
3	10.26	10.21	130	0.8	-0.20
4	9.93	10.31	238	0.1	1.67
5	10.34	10.31	224	0.9	-0.13
6	9.60	10.08	185	0.05	1.94

## 5.6 DISCUSSION

### 5.6.1 The prevalence of *T. brucei* in cattle

The initial prevalence of *T. brucei* in Tororo as determined by PCR was significantly higher ( $p = 0.02$ ) in the animals that were to be treated than those that were not intended for treatment, 11.2% compared to 3.8% (Figure 38a). However, in Busia the initial prevalence of *T. brucei* in the treatment and non treatment villages was not different, 13.8% vs. 10.0% ( $p > 0.1$ ). As has been mentioned in the previous Chapter, the overall initial prevalence was much higher than previously recorded in cattle in this area (Magona and Mayende, 2002).

While there was no statistically significant difference in the prevalence of *T. brucei* between treated and untreated cattle in Tororo up to month five, in Busia the prevalence was lower in the treated cattle than the untreated cattle in the second, third, fourth and eighth months in Busia (3.6% vs. 13.9%, 2.7% vs. 6.7%, 0% vs. 9.6% and 0% vs. 11.6% respectively, in all the months ( $p < 0.01$ )). However, in the sixth month the results showed a significant increase in prevalence, 54.8%, in the treated cattle in Tororo compared to 12.7% in the untreated animals ( $p < 0.0001$ ; Figure 38a). Similarly, there was an increase in prevalence up to 25.5% in Busia cattle compared to 9.5%,  $p < 0.005$  in the sixth month, Figure 38 (b). This upsurge in prevalence in month six was however not evident with microscopy. While the upsurge in prevalence in the sixth month following treatment in both districts is particularly interesting as it occurred in different months in the two districts, its cause is unclear. However, one possible explanation is that this could have been due to the elimination of acquired resistance to infection by trypanosomes in exposed non-patent animals that could have been perturbed following prophylactic treatment. A similar situation had been demonstrated in cattle treated against onchocerciasis with ivermectin, (Njongmeta, *et al.*, 2004). However, further studies need to be conducted involving regulated vector activities in order to determine the possible cause of this rise in prevalence following treatment.

The results also showed a rise in prevalence in the treated cattle to 25.8% compared to 9.0%,  $p = 0.004$ , in the untreated cattle, in the thirteenth month in Tororo, Figure 38(a).



There was a similar rise in prevalence in Busia, 44.4% in the treated compared to 17.2%,  $p < 0.005$ , in the untreated animals, but in the tenth month, Figure 38 (b).

When the data from the two districts was combined the results showed that the initial prevalence of treatment cattle was significantly higher than the no treatment ones, 12.5% vs. 6.9%,  $p = 0.02$ . However, in the second and third months following treatment, the prevalence dropped more significantly in the treated cattle than in the untreated ones,  $p < 0.01$ , Figure 38 (c). As had been observed, in the sixth month the prevalence was significantly higher in the treated cattle than in the untreated ones, 41.9% compared to 11.2%,  $p < 0.0001$ . Apart from the few months in which there was an upsurge in prevalence in the treated cattle, the prevalence was generally lower in the treated cattle than in the untreated ones,  $p < 0.01$ , Figure 38 (c), suggesting that prophylactic treatment reduced the prevalence by either clearing the existing infection or conferring some protection against new infections.

A two-sample T-test was used to test for equality of treatment and no treatment in cattle under infection with *T. brucei* (at 95% CI (-0.1503 and -0.0198),  $F = 4.50$  on 44 and 52 d.f., SE for difference of means = 0.03275,  $n = 320$  in each group,  $p < 0.001$ ) indicating a strong evidence of unequal sample variances. When the analysis was done to test whether the mean prevalence of *T. brucei* in treated animals is equal to that of untreated animals, it was observed that there was significant difference between the mean month on month prevalence of *T. brucei* in treated animals and the untreated ones ( $p = 0.011$ ) as the mean overall prevalence was lower in the treated animals than in those that received no treatment. This is consistent with the previous finding by Magona, *et al.* (2004) where it was shown that isometamidium chloride had protective efficacy against *T. brucei* infections in cattle.

The prevalence of *T. brucei* in individual villages of Tororo and Busia districts was quite variable (Figure 39). In the villages of Tororo, the initial prevalence determined in the month of July was 6.3% in Bunghaji, 3.8% in Ojilai, 8.6% in Hitunga and 13.8% in Magoje. In the third and fourth months following treatment the prevalence dropped to

below 5% in all the villages. However, in the month of November, the prevalence rose to 18.2% in Bunghaji but dropped to 0% the following month. The prevalence rose to 10% in Ojilai, 23.1% in Hitunga and 1.4% in Magoje in the month of November. In the month of December the prevalence rose to 18.2% in Ojilai. Hitunga recorded the highest prevalence of 42.79% in the month of December and 74.7% in the month of January. In Magoje the prevalence remained at 9.4% in December but rose to 30.0% in the month of January, Figure 39 (a) despite treatment. The prevalence remained at 0% in Bunghaji and dropped to 12.7% in Ojilai in the same month of January. In the villages of Busia, the initial prevalence determined in the month of October was 7.5% in Buyimini, 12.5% in Sitengo, 13.8% in both Kubo and Magoje. In the following month the prevalence dropped to 0% in Buyimini and Kubo but remained at 12.8% and 5.3% in Sitengo and Nanjeho villages. The highest prevalence of 38.3% was recorded in Kubo in the month of April while in Sitengo the highest prevalence of 22% was recorded in the month of March, Figure 39 (b).

Out of the cattle that were found infected with *T. brucei* in Tororo 104 were re-screened for *T. b. rhodesiense* by *SRA* gene -targeting primers. Out of these cattle 23 were from the baseline sampling while 7 were from the second month after treatment, 2 from the third, 9 from the fourth, 38 were from the fifth while 29 were from the sixth month. Five animals (25%, CI 8.7 and 49.1), from the baseline samples were found positive for *SRA*, indicating that they were *T.b. rhodesiense* (Welburn, *et al*, 2001; Gibson, *et al.*, 2003). This represented a ratio of 1 out of 4 *T. brucei* positive animals being *T.b. rhodesiense*. There were no positives either with TBR or *SRA* primers up to the fifth month. However, out of the 29 samples screened from the sixth month, 17 re-amplified with *T.brucei* primers and four (23.8%, CI 6.8 and 49.9), were detected positive with *SRA* primers. Once again the ratio was one out of four. Moreover, all the five samples were from the animals that had received isometamidium treatment. The results indicate that the overall prevalence of *T. b. rhodesiense* was 24% of the *T. brucei* positives. The overall minimum prevalence of *T.b. rhodesiense* in the 104 samples screened was 24% (CI 11.8 and 41.2), which was not significantly different from the 18% found by Welburn, *et al.* (2001).

### 5.6.2 The prevalence of *T. congolense* in cattle

The initial prevalence of *T. congolense* in treated and untreated cattle from Tororo and Busia districts was found to be 16.4%, much higher than previously reported in this area, (Ford, 1971). In Tororo the initial prevalence in the treatment group of cattle was significantly higher than in the no treatment animals (18.6% compared to the 6.3% in the animals that were not to be given any treatment,  $p = 0.002$ ). However, in Busia the initial prevalence in the treatment group of cattle and non treatment group was not different (21.9% compared to 19.4%,  $p > 0.1$ ). The pattern was similar to what was observed with *T. brucei*, where the initial prevalence was different between the groups in Tororo but not in Busia. However, in the months following treatment there was no significant difference between the two groups in Tororo up to the fourth month when the prevalence in the treated cattle dropped much lower than in the untreated animals, 1.3% vs. 26.2%,  $p < 0.0001$ , (Figure 40a). A similar drop in prevalence in the treated animals was observed in Busia (0.7% vs. 7.6%,  $p = 0.01$ ) but in the second month following treatment.

However, in the second month the prevalence was lower in the treated cattle than in the untreated ones. The highest rise in prevalence in the treated group in Busia cattle, 27.6%, was in the fourth month compared to 6.1% in the untreated cattle,  $p < 0.0001$ , Figure 40 (b). When the data from the two districts was combined the results showed that the initial prevalence of treatment cattle was significantly higher than the no treatment ones, 20.2% vs. 12.9%,  $p = 0.02$ . However, in the second and fifth months the prevalence was lower in the treated cattle than in the untreated ones, 1.1% and 0.4% vs. 4.5% and 4.4%,  $p < 0.03$ , Figure 40 (c).

When the monthly mean prevalence of *T. congolense* infections in cattle with and without isometamudium chloride treatment was pooled the overall initial mean prevalence, 20.2% and 12.9% in the treated and untreated animals respectively. There was a drop in the mean prevalence to 1% in the first month in both groups of cattle. The first mean peak prevalence in both groups were not significantly different, 16.8% in the treated and 13.2%,  $p > 0.05$  in the untreated animals, Figure 40 (c). These results suggest that treatment of animals offered

no protection against *T. congolense* infection, even though the number of animals was increased considerably. The results were confirmed when the analysis was done to test the null hypothesis that the mean prevalence in untreated is equal to the mean of treated (Test statistic  $t = -0.77$  on 50 d.f.  $p = 0.447$ ) indicating that there was no significant difference in prevalence between treated and untreated animals. These results are consistent with studies where drug resistance has been observed (Afewerk, *et al.*, 2000, Rowlands, *et al.*, 2001).

In the individual villages of Tororo, the initial prevalence determined in the month of July was 3.8% in Bunghaji, 8.9% in Ojilai, 17.3% in Hitunga and 20% in Magoje. In the second and third months following, treatment the prevalence dropped below 5% in all the villages. However, in the month of October, it rose to 6.1% in Ojilai and 15.0% in Hitunga. In the month of November the prevalence rose to 38.2% in Bunghaji, 17.3% in Ojilai, but only to 2.6% in Hitunga and 0% in Magoje. Subsequently, the prevalence remained at 0% and less than 3% in Ojilai, Magoje and Hitunga but rose to 3.8% in December and 7.5% in February in Bunghaji, Figure 41 (a).

In the villages of Busia, the initial prevalence determined in the month of October was 8.8% in Buyimini, 30.0% in Sitengo, 18.8% in Kubo and 25.0% in Nanjeho. In the following month prevalence dropped to 0% in all the villages. In the month of December the prevalence rose again to 7.0% and 8.2% in Buyimini. In the month of January the prevalence rose to 17.1% and in the month February the prevalence was 10.2% in Buyimini and up to 30.0% in Nanjeho, Figure 41 (b).

### **5.6.3 The prevalence of *T. vivax* in cattle**

The prevalence of *T. vivax* in treated and untreated cattle from Tororo and Busia districts show that in Tororo the initial prevalence in the treatment group of cattle was significantly higher than in the no treatment animals, 23.6% vs. 13.3%,  $p = 0.01$ . However, the results showed that there was no significant difference in the subsequent months up to the fourth month when the prevalence in the treated cattle was lower than in the untreated animals, 0.7% vs. 3.1%,  $p < 0.001$ . In the subsequent three months, there was no significant

difference in prevalence between the two groups of cattle, Figure 42 (a). In Busia the initial prevalence in the treatment group was lower than in the non- treatment group of cattle, 6.3% vs. 20.6%,  $p < 0.001$ . This difference persisted in the following month where the prevalence in the treated cattle was 0.7% vs. 5.2% in the treated animals,  $p = 0.04$ . Subsequently, there was no difference in prevalence in the two groups up to the fifth month when the prevalence remained at 1.8% in the treated cattle but significantly rose to 12.7%, ( $p < 0.001$ ) in the untreated animals (Figure 42b).

When the data from the two districts was combined the results showed that the initial prevalence of treatment cattle was not different from the no treatment ones, 15.0% vs. 17.0%,  $p = 0.5$ . However, in the following month the prevalence was lower in the treated cattle than the untreated, 0.3% vs. 2.8,  $p = 0.04$ . Subsequently there was no difference in the prevalence between the two groups of animals, Figure 42 (c). This was confirmed when the analysis was done to test the null hypothesis that the mean of prevalence in untreated is equal to the mean of treated and the result showed a test statistic,  $t = -0.50$  on 18 d.f.,  $p = 0.623$ , indicating that there was no significant difference in prevalence between treated and untreated animals.

The initial prevalence in the villages of Tororo ranged from 6.3% in Bunghaji, 20.3% in Ojilai, 11.1% in Hitunga and 36.3% in Magoje. In September the prevalence was 8.2% in Bunghaji, but dropped to 1.9% in Ojilai, 6.6% in Hitunga and 0% in Magoje. However, the prevalence rose to 25.8% in Bunghaji, 7.6% in Ojilai, 40.0% in Hitunga and 6.4% in Magoje in the month of October. In the month of November the prevalence dropped to below 4% in all the villages but rose to 5.8% in Bunghaji, 13.8% in Ojilai, 32.0% in Hitunga and 6.3% in Magoje, Figure 43 (a). In the villages of Busia, the initial prevalence of *T. vivax* determined in the month of October was 15% in Buyimini, 26.3% in Sitengo, 5.0% in Kubo and 7.5% in Magoje. In the following month the prevalence dropped to 0% in Buyimini and Kubo, 10.3% in Sitengo and 1.3% in Nanjeho. In January the prevalence rose to 14.5% in Buyimini, 13.7% in Sitengo, 7.9% in Kubo and Nanjeho, Figure 43 (b).

#### 5.6.4 The prevalence of any trypanosome species in cattle

The results show that in Tororo the initial prevalence in the treatment group of cattle was significantly higher than in the no treatment animals, 42.2% vs. 21.5%,  $p = 0.0001$ . The results showed that there was no significant difference in the subsequent months up to the fourth month at which point the prevalence in the treated cattle was lower than in the untreated animals, 14.7% vs. 34.6%,  $p < 0.0001$ . However, in the sixth month, the prevalence was significantly higher in the treated cattle than the untreated ones, 56.3% compared to 16.4%,  $p < 0.0001$ , Figure 44 (a).

In Busia there was no significant difference in initial prevalence in the treatment and no treatment groups, 36.3% vs. 41.9%,  $p = 0.76$ . However, in the first, second and fifth months following treatment, the prevalence was significantly lower in the treated cattle than the untreated ones, 3.4%, 8.8% and 8.8% vs. 11.1%, 22.9 and 26.3% respectively,  $p < 0.05$ . In the sixth month the prevalence in the treated cattle rose significantly higher than the in the untreated animals, 40.6% compared to 22.9%,  $p = 0.01$ , Figure 44 (b).

When the data from the two districts was combined the results showed that the initial prevalence of any trypanosome in the treatment cattle was not different from the no treatment ones, 39.3% vs. 31.8%,  $p = 0.54$ . However, in the following two months the prevalence was reduced in the treated cattle than the untreated, 1.7% and 6.3% vs. 6.0 and 15.9%,  $p < 0.03$ . Subsequently there was no difference in the prevalence between the two groups of animals up to the sixth month when the prevalence in the treated cattle rose significantly higher than the untreated animals, 49.4% vs. 19.5%,  $p < 0.0001$ , Figure 44 (c). This overall difference was largely due to the upsurge in the prevalence of *T. brucei* in the sixth month in both districts.

When cattle were examined by microscopy the prevalence of any trypanosome species from the two districts it was found that that in Tororo the prevalence of any trypanosome species was significantly reduced in the third month, 0.6% in the treatment cattle than the 6.1% in the untreated animals, ( $\chi^2 = 5.4$ ,  $p = 0.02$ ). The reduction was also evident in the



eighth month, 1.7% in the treated compared to 10.4% in the untreated animals, ( $\chi^2 = 6.3$ ,  $p = 0.01$ ) and in the fourteenth month, 0.9% in the treated compared to 7.9% in the untreated animals, ( $\chi^2 = 4.5$ ,  $p = 0.03$ ) months, Figure 45 (a). However, in Busia the prevalence was significantly different in the fourth month, 11.4% in the treated compared to 4.4% in the untreated animals, ( $\chi^2 = 13.6$ ,  $p < 0.001$ ) and the sixth months, 10.4% in the treated compared to 4.8% in the untreated animals, ( $\chi^2 = 5.6$ ,  $p = 0.02$ ), Figure 45 (b). Overall, the prevalence was not different in treated and untreated cattle from both districts except in the first month, 0% in treated and 4.6% in the untreated animals ( $\chi^2 = 12.2$ ,  $p = 0.001$ ), as was observed in Figure 45 (c).

While some protective effect of isometamidium chloride treatment of cattle on the prevalence of the three trypanosome species in both districts was apparent as determined by PCR amplification, this was not evident with microscopy, Table 23 (a) and (b). The results with microscopy would lead to the conclusion that there was complete treatment failure in both district, whereas it is clear that there was some protective effect in areas where the prevalence was low. Furthermore, microscopy failed to demonstrate the negative effect which treatment had in both districts in month six against *T. brucei* infections as was evident from the PCR analysis.

### **5.6.5 The incidence of trypanosome species in cattle from Tororo and Busia districts**

In Tororo there were 19 new infections in the treated group compared to 12 in the untreated group in the third month following treatment. This continued to rise to 26 cases in the treated cattle compared to 19 in the untreated group in the sixth month. There were 11 reccuring cases from the previous postives during this period, Table 24 (a). In Busia there were 8 new cases in the treated group compared to 6 in the untreated and this continued to rise to 28 new cases in the treated group compared to 3 in the untreated, Table 24 (b). These results indicate that the rise in prevalence in the treated group could be attributed to lack of protection or loss of natural resistance by cattle against new infections rather than drug breakthrough.

After treatment of cattle the incidence of *T. congolense* in Tororo was highest in the third and fourth months, 18 and 29 new cases respectively. Of the 18 new cases in the third month 5 were in the untreated villages, 8 were in the treated villages while 5 were recurring after previously being detected. Of the 29 new cases in the fourth month 27 were in the untreated villages, while 2 were recurring after previously being detected. The incidence was lowest in the first and second months and then in the sixth month, Table 25 (a). The incidence of *T. congolense* in cattle from Busia villages was highest in the months of third and fourth months, 19 and 43 respectively. Of the 19 new cases in third month, 3 were in the untreated villages, 13 were in the treated villages while 3 were recurring after previously being detected. Of the 43 new cases in fourth month, 8 were in the untreated villages, 30 were in the treated villages while 5 were recurring after previously being detected. The incidence was lowest in the first and fifth months, (6), Table 25 (b). These results suggest that treatment of cattle provided protection against infection in Tororo where the initial prevalence was low but not in Busia where the initial prevalence of *T. congolense* was high.

After treatment the incidence of *T. vivax* in cattle from Tororo villages was highest in the fourth and sixth months, 57 and 38 new cases respectively. Of the 57 new cases in the fourth month, 19 were in the untreated villages, 32 were in the treated villages while 6 were recurring after previously being detected. Of the 38 new cases in sixth month, 10 were in the untreated villages, 16 were in the treated villages while 12 were recurring after previously being detected, Table 26 (a). In Busia villages, incidence of *T. vivax* in cattle was highest in the third, and sixth months, 26 and 33 respectively. Of the 26 new cases in the third month, 15 were in the untreated villages, 9 were in the treated villages while 2 were recurring after previously being detected. Of the 33 new cases in the sixth month, 20 were in the untreated villages, 12 were in the treated villages while 1 was recurring after previously being detected, Figure 26 (b). These results indicate that treatment of cattle reduced the prevalence below detectable levels, but did not confer protection for new infections.

When the data from all the villages in Tororo and Busia districts were pooled together and the comparison made between treated and untreated animals, the mean prevalence of any trypanosome species in cattle dropped from the initial 15% to less than 3% in both groups. The mean prevalence rose to a first peak of 10% in the third month corresponding to 8% in the untreated group,  $\chi^2 = 0.7$ ,  $p = 0.4$  and in the sixth month, the mean prevalence increased to 14% in the treated group of animals, corresponding to 7% in the untreated group,  $\chi^2 = 8.8$ ,  $p = 0.003$ . This was largely because of the effect of increased prevalence of *T. brucei* in all locations, as had been found, Figure 38. Moreover, when a test of the null hypothesis that the mean of mean prevalence with untreated was equal to that of treated cattle, was done, it was observed that a test statistic  $t = -0.35$  on 39.46 d.f. with probability = 0.731, indicating that there were no significant differences in the mean prevalence between treated and untreated animals, leading to the conclusion that treatment of cattle with isometamidium chloride did not confer protection to animals under trypanosome infection in areas where the prevalence of trypanosomes is high.

However, there is need to conduct further studies that would include the vector activities and quantitative measurements of trypanosome challenge in this area. It is important to note that there were no data regarding tsetse density and trypanosome transmission dynamics during the period of this study. This could provide some insight as to whether there was clear drug resistance in the study area or if it was simply due to high transmission rate that could have overwhelmed the effect of treatment as was observed with *T. vivax* in Tororo district. In conclusion, these results suggest that treatment of cattle with isometamidium chloride did not confer any protection against infections by any of the three species of trypanosomes, as determined by either PCR amplification or microscopic examination. Instead, the upsurge of *T. brucei* prevalence in the treated animals, as was found with PCR amplification, appears to complicate the estimation of the true effect of the drug.

#### **5.6.6 Effect of treatment of cattle with isometamidium chloride on anaemia**

The analysis of the effect of Samorin treatment of cattle on anaemia assumed that anaemia could have only been due to trypanosomiasis. The results of the monthly mean haemoglobin concentration in treated and untreated cattle from Tororo (Table 27) indicate that there was no significant difference between treated and untreated animals for the first five months ( $p > 0.05$ ). However, in the sixth month, the mean haemoglobin concentration was higher in treated animals, 10.69g/dl compared to 10.03g/dl in the untreated animals,  $t = -2.08$ , d.f. = 154 ( $p = 0.04$ ). The results in Busia were also similar up to the fifth month. However, in the sixth month the mean haemoglobin concentration was lower in treated animals, 9.60g/dl compared to 10.08g/dl in the untreated animals,  $t = 1.94$ , d.f. = 185 and  $p = 0.05$ , Table 28. These results imply that treatment with isometamidium chloride does not have any significant effect on the state of anaemia caused by trypanosomiasis. However, it should be noted that anaemia could have been due to other pathogens such as *Anaplasma*, affecting the animals which were not targeted by treatment. These findings are consistent with the results of a study carried out by Efewerk, *et al.*, (2000), in which they observed that the mean PCV of East African zebu cattle treated with isometamidium chloride did not differ from those not given treatment for up to three months. In their study, the animals were also kept under traditional husbandry and therefore there was a possibility that the animals had infections with other anaemia inducing pathogens.

## CHAPTER SIX

### 6 THE EFFECT OF DRUG INTERVENTION AGAINST INFECTIONS WITH TRYPANOSOMES IN CATTLE FROM SE UGANDA

#### 6.1 INTRODUCTION

Control of trypanosomiasis in Uganda is currently done by either controlling the tsetse vector or by the use of both curative diminazine aceturate (Berenil) and prophylactic isometamidium chloride (isometamidium chloride) drugs. However, these two drugs have been in use for over 40 years and there have been reports of wide-spread resistance by various species of trypanosomes, in many parts of Africa including Uganda (Mbwabo, *et al.*, 1988; Boid, *et al.*, 1989; Stevenson, *et al.*, 1995; Afewerk, *et al.*, 2000). In Uganda drug resistance was reported as early as 1971 (Mwambu and Mayende, 1971; Enyaru, *et al.*, 1998). Drug resistance by a number field isolates of trypanosome has been demonstrated in the laboratory (Peregrine, *et al.*, 1991; Peregrine, 1994) and recently a standardized single-dose-mouse-based test has been developed for testing drug resistance in trypanosome isolates from different areas (Eisler, *et al.*, 2001).

Epidemiological data used in determining drug resistance are usually collected using the insensitive and non-specific microscopic examination of blood samples from cattle. The use of molecular techniques has been shown to be highly sensitive and specific in identifying the various species and types of trypanosomes in cattle both in the laboratory (Moser, *et al.*, 1989; Masiga, *et al.*, 1992; Masake, *et al.*, 1997; Majiwa, *et al.*, 1993; Majiwa and Otieno, 1990; Gashumba, *et al.*, 1988; Majiwa, *et al.*, 1994) as well as in the field (Duvallet, 1999; Desquesnes and Davila, 2002). The vast areas covered in this study and the quality and quantity of the data obtained has allowed the analysis and comparison of the effect of prophylactic treatment against the three main species of trypanosomes affecting cattle over a period of six months. The data has also provided a quantitative way of determining the time to re-infection of cattle with the various trypanosome species following treatment with isometamidium chloride. A survival analysis was done to determine the time to infection/re-infection of cattle following prophylactic treatment with isometamidium

chloride against the three species of trypanosomes that infect cattle in SE Uganda. This method has recently been used to assess the diagnostic test performance on Trypanosomiasis under natural field challenge, (Greiner, *et al.*, 2001). More recently the method has been used in assessing drug resistance of trypanosomes in cattle in western Ethiopia, (Tewelde, *et al.*, 2004) and Magona *et al.*, (2004) have applied the analytical method in the study of protective efficacy of isometamidium chloride and diminazene aceturate against natural trypanosome species infection in cattle under a suppressed tsetse population in Uganda.

### **6.1.1 Objective of the study in this Chapter**

The main objective of this Chapter was to further assess the effect of prophylactic treatment of cattle with isometamidium chloride on the infection by trypanosomes. To achieve this objective, the data obtained by PCR amplification of various trypanosome species in cattle, as has been described in Chapter 3, was subjected to survival analysis which is a more precise method. This method allowed for the examination of the effect of the drug on the prevalence of trypanosomes at designated time points.

The effect of diminazene aceturate treatment of cattle on the infection of cattle with any trypanosome species was also assessed. Based on the hypothesis that diminazene aceturate, one of the drugs used in the treatment of trypanosomiasis in cattle clears the parasites but does not confer protection against new infections, a simple linear model was used to assess the effect of the drug on the infection of cattle with trypanosomes.

## **6.2 MATERIALS AND METHODS**

The study areas covering eight villages, four from Tororo and four from Busia districts of South- East Uganda have been described in Chapter 2. The geographical locations of the study villages, rainfall patterns and the criteria used in selecting the villages have also been described in Chapter 2. The villages were randomly assigned to treatment groups, each treatment replicated in two villages of each district as indicated in chapter 2. Diminazene treatment of cattle was done during the visits when the haemoglobin levels dropped to



8mg/dl or below as estimated by the HaemoCue. The cattle were screened for the three species of trypanosomes by PCR amplification as described in Chapter 3.

### 6.2.1 Survival analysis

Survival analysis is concerned with studying the time between entry to a study and a subsequent event. Originally the analysis was concerned with time from treatment until death, hence the name, but survival analysis is applicable to many areas as well as mortality and in this case, to the time-to-trypanosome infection. The analysis has been applied in assessing isometamidium chloride resistance of trypanosomes in cattle in western Ethiopia, (Tewelde, *et al.*, 2004). The survival statistical analysis was done using GraphPad Prism software version 3.1, to determine the effect of isometamidium chloride treatment of cattle on the infection by each of the three species of trypanosomes. All comparisons in the survival analyses between isometamidium chloride- treated and untreated groups of cattle were done by using the log rank Chi-square test which is a non- parametric test that does not require the sample data to be normally distributed, although it assumes that the variables were normally distributed in the population from which the representative animals were drawn. The log rank test is so called because it is related to a test that uses the logarithms of the ranks of the data.

The assumptions used in this test are:

1. That the survival times are ordinal or continuous.
2. That the risk of an event in one group, (in this instance, the detection of trypanosome by PCR amplification, also referred to as infection) relative to the other does not change with time.

The comparisons were done at 95% C.I. and presented as Kaplan-Meier Survival curves (Peto, *et al.*, 1977). The proportion of animals,  $S(t)$ , surviving beyond any follow up time ( $t$ ) was estimated by the formula:

$$S(t) = \frac{(r_1 - d_1)}{r_1} \times \frac{(r_2 - d_2)}{r_2} \dots \times \dots \frac{(r_p - d_p)}{r_p}$$

where  $t_p$  is the largest survival time less than or equal to  $t$  and  $r_i$  is the number of animals alive just before time  $t_i$  (the  $i$ th ordered survival time),  $d_i$  denotes the number who died at time  $t_i$  where  $i$  can be any value between 1 and  $p$ . For censored observations  $d_i = 0$ .

The following criteria were adopted for filtering the data for the Survival analysis in both Tororo and Busia districts:

- All animals that were found to be infected at the baseline, in both the treated and untreated villages just before ISMM treatment were excluded from the analysis since the presence of trypanosome infection was regarded to be the determinant hazard.
- All animals treated with diminazene in the course of the study were excluded from the analysis after such treatment, since the treatment altered the hazard rate and changed the status of these animals while it does not relate in this case to the hazard outcome.
- All animals which were recruited later in the course of study were included in the analysis subject to the following assumptions:
  1. That the monthly hazard rate was the same and applied to all animals in each group equally.
  2. That these late recruits were all uninfected throughout the time prior to their recruitment.
- All animals that were missing from any time point in the study were included in the analysis provided they were uninfected prior to the time they were not sampled as well as at the start of the study.
- All animals that remained uninfected throughout the study without intervention with diminazene treatment were censored.

### **6.2.2 Time to re-infection of cattle with trypanosomes following isometamidium chloride treatment.**

During the screening of cattle in the baseline study in both Tororo and Busia villages 40 animals were found to be infected with *T. brucei*, 65 with *T. congolense* and 48 with *T. vivax* out of a total of 640 animals examined by PCR amplification with species and type-specific primers prior to treatment with isometamidium chloride. A corresponding number of animals were selected from a group that had no infection but received the same treatment, in the same villages, by using a random Table (Woodward, 1999) and monitored as controls in the same way. Following treatment with isometamidium chloride both groups of animals were screened for the three trypanosome species for six months to determine the time to re-infection.

The 40 animals from the treatment villages in the two districts which had detectable *T. brucei* prior to treatment were pooled together and the cumulative proportion of the animals that had detectable trypanosomes by PCR amplification at various time points was determined. Sixty-five animals that had been detected with *T. congolense* from the treatment villages in the two districts were also pooled together and the proportion of the animals that had detectable trypanosomes by PCR amplification at various time points was determined. Forty-eight animals from the treatment villages in the two districts that had been detected with *T. vivax* were similarly pooled together and the proportion of the animals that had detectable trypanosomes by PCR amplification at various time points was determined. A total of 153 animals from the treatment villages in the two districts, which had any of the three species of trypanosomes detected prior to treatment, were pooled together and the proportion of the animals that had any of the three trypanosome species detectable by PCR amplification at various time points was determined. A corresponding equal number of animals from the same treatment villages but had no detectable trypanosomes prior to treatment were also monitored for each trypanosome species as controls.

### 6.2.3 Analysis of the effect of treatment of cattle with diminazene aceturate against trypanosome infection

In the course of the study, animals which showed signs of infection with trypanosomes based on either clinical examination or anaemia as determined by a minimum cut-off point of 8g/dl haemoglobin were treated with diminazene aceturate as indicated in Chapters 2 and 3. These animals were used in assessing the effect of treatment on infection and re-infection with trypanosomes. A total of 689 samples were analysed for the effect of the drug on *T. brucei* infection, 207 for *T. congolense* and 208 for *T. vivax*. The data used for *T. brucei* analysis were from a period of 16 months while for those for *T. congolense* and *T. vivax* were for a period of 8 months. Combined data from both districts and collected by both microscopy and PCR amplification were subjected to the analysis.

A simple linear model was developed to test the following hypotheses:

- That diminazene clears, but does not prevent infection or re-infection of cattle with trypanosomes by 28 days after its administration.
- That the clearance effect, if it occurs, would be more apparent with microscopic examination but not by PCR, since the sensitivity of PCR has been shown to be superior to that of microscopy in Chapter 4. This would suggest that diminazene treatment lowers the burden of infection detectable by microscopy but does not clear the parasites since they can still be detected by PCR.

The significant terms and sample sizes used in the model are listed in Table 29.

Table 29: Significant terms for models 1 and 2. (Best random formula = Animal ID)

	Sample size	Significant terms	F-value	P-value
Microscopy	7828 observations on 846 cattle	Microscopy diagnosis for month x	$F_{1,6980} = 9.3855$	0.0022
		Diminazene	$F_{1,6980} = 30.1911$	<.0001
PCR	5481 observations on 715 cattle	PCR diagnosis for month x	$F_{1,4764} = 8.8389$	0.0030
		Diminazene	$F_{1,4764} = 4.0470$	0.0443

6.3 RESULTS

6.3.1 The effect of isometamidium chloride (ISMM) treatment of cattle against *T. brucei* infection

Comparative analyses of the survival curves of *T. brucei* infection data from the treated and untreated animals from Tororo over a period of 161 days is shown in Figure 47. The result indicate that there were no significant differences between the two groups, ( $\chi^2 = 3.18$ ,  $p = 0.074$ ). Out of the initial 136 cattle at risk in the treated group and 163 in the untreated group 65 from the treated group were censored while 132 from the untreated group were censored. The results also indicate that at 161 days, 63% of the animals in the treated group remained uninfected while 49% in the untreated group remained uninfected, Table 30.

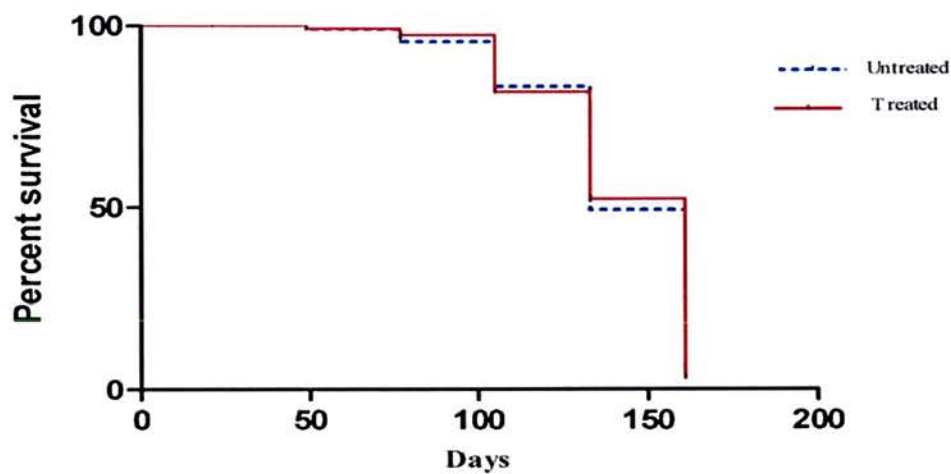


Figure 47: Kaplan-Meier Survival plot for *T. brucei* infection in ISMM treated and untreated cattle from the villages of Tororo district.

Table 30: Animals at risk of infection with *T. brucei* and survival proportion of cattle from Tororo villages

<u>Days</u>	<u>Animals at risk</u>		<u>Survival proportions</u>	
	<u>Treated</u>	<u>Untreated</u>	<u>Treated</u>	<u>Untreated</u>
0	136	163	100	100
21	136	163	100	100
49	121	106	99	99
77	115	85	97	96
105	99	70	82	83
133	61	44	52	49
161	32	11	3	49

Comparative analyses of the survival curves of the *T. brucei* infection data over a period of 161 days from the treated and untreated animals from Busia is shown in Figure 48. The results indicate that there were no significant differences between the two groups, ( $\chi^2 = 2.44$ ,  $p = 0.118$ ). The analysis shows that out of the initial 122 animals at risk in the treated group 90 were censored while out of the 107 animals in the untreated group 86 were censored. The results also indicate that at the end of the study, 41 animals in the treated group were at risk compared to 8 in the untreated group. The results also show that 46% of the animals in the treated and 63% in the untreated group remained uninfected up to day 161, Table 31.



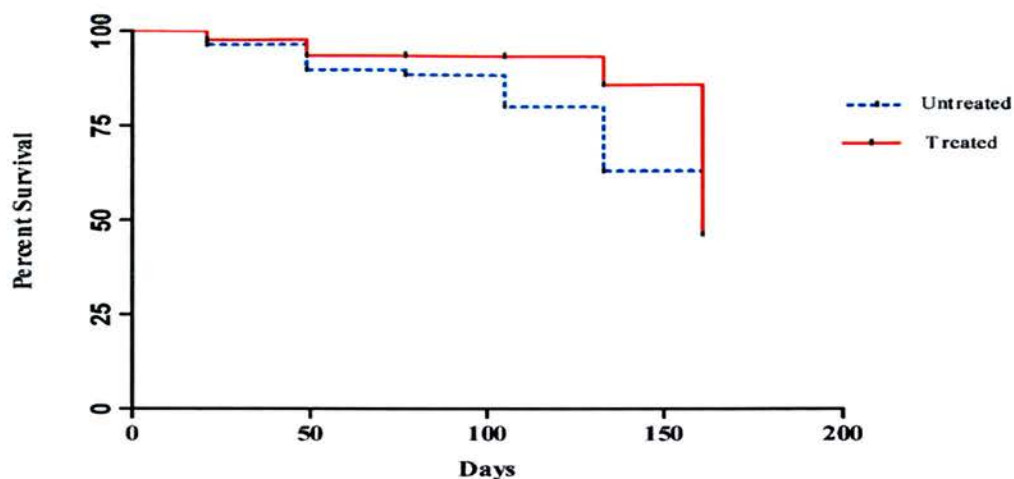


Figure 48: Kaplan-Meier Survival plot for *T. brucei* infections in ISMM treated and untreated cattle from the villages of Busia district.

Table 31: Animals at risk of infection with *T. brucei* and survival proportion of cattle from Busia villages

Days	<u>Animals at risk</u>		<u>Survival proportions</u>	
	Treated	Untreated	Treated	Untreated
0	122	107	100	100
21	122	107	98	96
49	116	87	93	90
77	97	66	93	88
105	82	43	93	80
133	62	28	86	63
161	41	8	46	63

When the data from the treated and untreated animals in the two districts was combined the comparative analyses of the survival curves of the *T. brucei* infection over a period of 161 days shows that there was no significant difference between the two groups, ( $\chi^2 = 0.0003$ ,  $p = 0.986$ ), Figure 49. The analysis shows that 22% of the animals in the treated group and 54% in the untreated group survived without infection up to 161 days, Table 32.

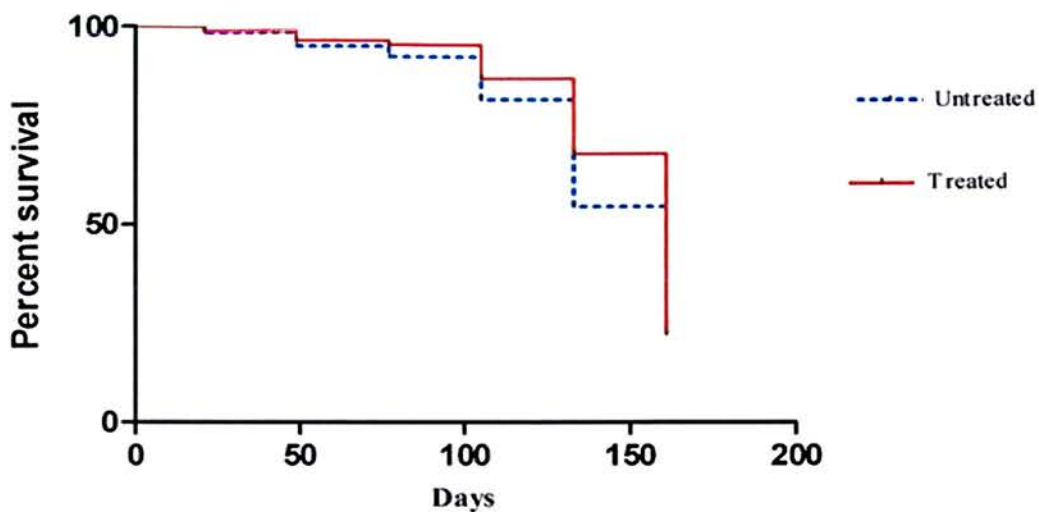


Figure 49: Kaplan-Meier Survival plot for *T. brucei* infections in ISMM treated and untreated cattle from both districts.

Table 32 Animals at risk of infection with *T. brucei* and survival proportion of cattle from both districts.

<u>Days</u>	<u>Animals at risk</u>		<u>Survival proportions</u>	
	<u>Treated</u>	<u>Untreated</u>	<u>Treated</u>	<u>Untreated</u>
0	258	269	100	100
21	258	269	99	99
49	237	192	96	95
77	212	150	95	92
105	181	112	87	82
133	123	72	68	54
161	73	19	22	54

**6.3.2. The effect of isometamidium chloride (ISMM) treatment of cattle against *T. congolense* infections.**

Comparative analyses of the survival curves of the *T. congolense* infection data from the treated and untreated animals in Tororo over a period of 161 days show that there was a significant difference between the two groups ( $\chi^2 = 16.85$ ,  $p = 0.0001$ ). The animals that received treatment had fewer infections compared to those that did not receive treatment, Figure 50. Out of the initial 351 cattle in the treated group and 231 in the untreated group 162 from the treated group were censored while 203 from the untreated group were censored. The results also indicate that 96% of the animals in the treated and 88% in the untreated groups survived without infection up to 161 days, Table 33.

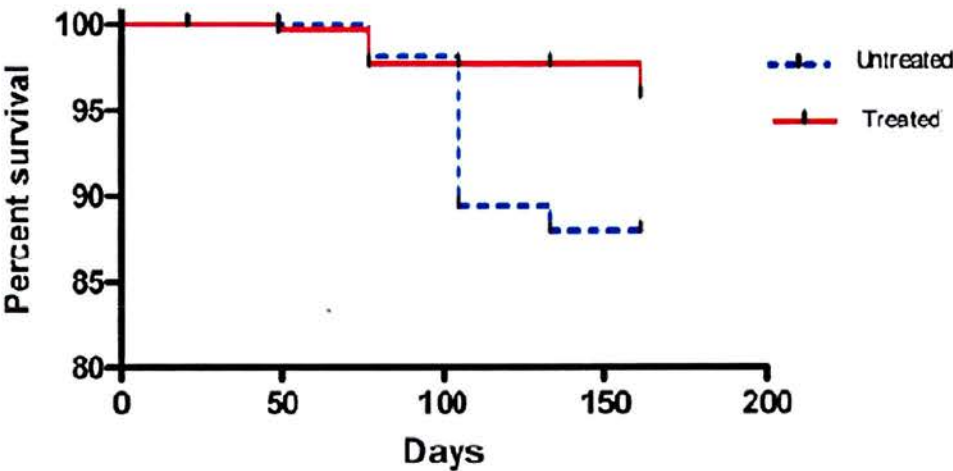


Figure 50: Kaplan-Meier Survival plot for *T. congolense* infections in ISMM treated and untreated cattle from the villages of Tororo district.

Table 33 Animals at risk of infection with *T. congolense* and survival proportion of cattle from Tororo villages.

Days	<u>Animals at risk</u>		<u>Survival proportions</u>	
	Treated	Untreated	Treated	Untreated
0	370	351	100	100
21	370	351	100	100
49	359	298	100	100
77	352	277	98	98
105	332	258	98	89
133	200	120	98	88
161	104	66	96	88

The analyses of the survival curves of the *T. congolense* infection data from the treated and untreated animals from Busia over a period of 161 days show that there were no significant differences between the two groups, ( $\chi^2 = 0.06$ ,  $p = 0.808$ ), Figure 51. The analysis shows that out of the initial 163 animals at risk in the treated group 130 were censored while out of the 142 animals at risk in the untreated group 117 were censored. The results also indicate that 74% of the animals in both groups survived without infection up to 161 days, Table 34.

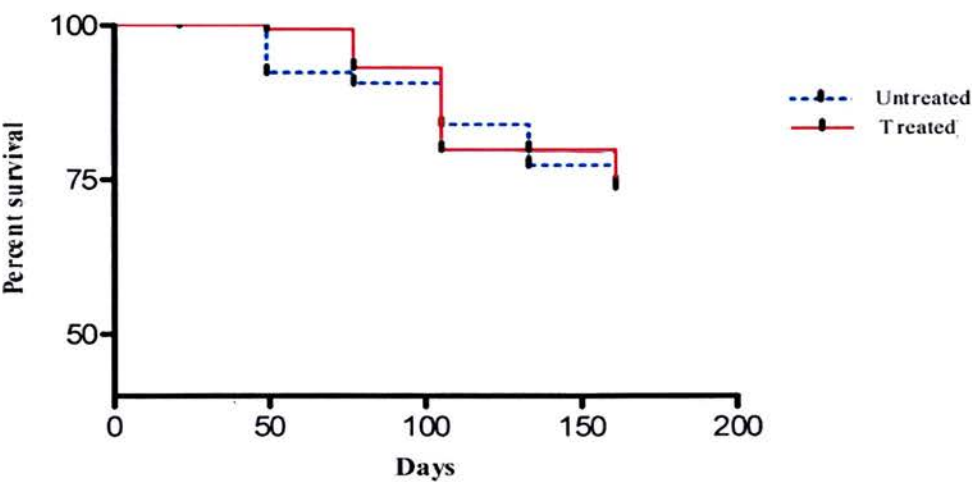


Figure 51 Kaplan-Meier Survival plot for *T. congolense* infections in ISMM treated and untreated cattle from the villages of Busia district.

Table 34: Animals at risk of infection with *T. congolense* and survival proportion of cattle from Busia villages.

<u>Days</u>	<u>Animals at risk</u>		<u>Survival proportions</u>	
	<u>Treated</u>	<u>Untreated</u>	<u>Treated</u>	<u>Untreated</u>
0	163	142	100	100
21	163	142	100	100
49	160	129	99	92
77	142	106	93	91
105	119	81	80	82
133	89	64	80	77
161	79	50	74	74

Comparative analyses of the survival curves of the *T. congolense* infection data from the treated and untreated animals from both districts over a period of 161 days show that there was a significant difference between the two groups, ( $\chi^2 = 13.80$ ,  $p = 0.0002$ ), Figure 52. The analysis shows that out of the initial 186 animals at risk in the treated group 165 were censored while out of the 141 animals at risk in the untreated group 105 were censored. The results also indicate that at 89% of the animals in the treated and 83% in the untreated groups remained uninfected up to 161 days, Table 35.

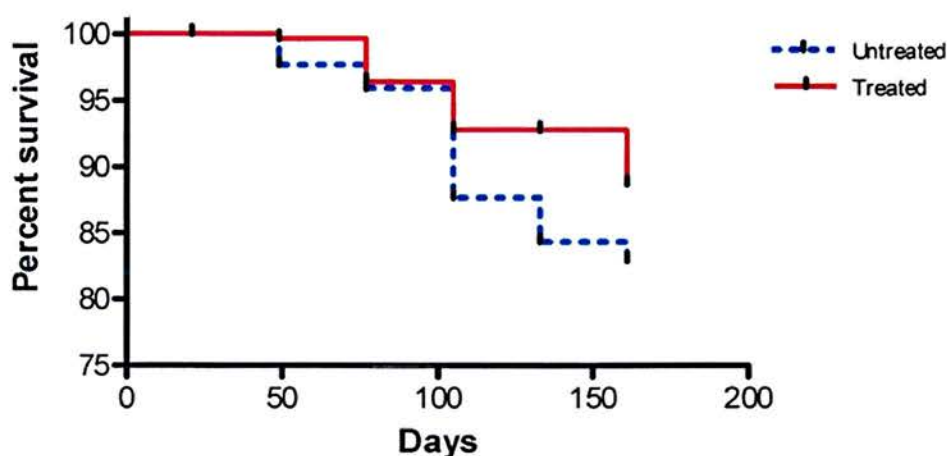


Figure 52: Kaplan-Meier Survival plot for *T. congolense* infections in ISMM treated and untreated cattle in both districts

Table 35 Animals at risk of infection with *T. congolense* and survival proportion of cattle from both districts.

Days	Animals at risk		Survival proportions	
	Treated	Untreated	Treated	Untreated
0	533	493	100	100
21	533	493	100	100
49	519	427	100	98
77	494	383	96	96
105	451	339	93	88
133	289	184	93	84
161	183	116	89	83

### 6.3.3 The effect of isometamidium chloride (ISMM) treatment of cattle against *T. vivax* infections.

Comparative analyses of the survival curves of the *T. vivax* infection data from the treated and untreated animals in Tororo over a period of 161 days show that there were no significant differences between the two groups, ( $\chi^2 = 2.40$ ,  $p = 0.12$ ), Figure 53. Out of the



initial 306 cattle at risk in the treated group and 381 in the untreated group 126 and 196 were censored in the respective groups. The results also indicate that 69% of the animals in the treated and 78% in the untreated groups survived without infection up to 161 days, Table 36.

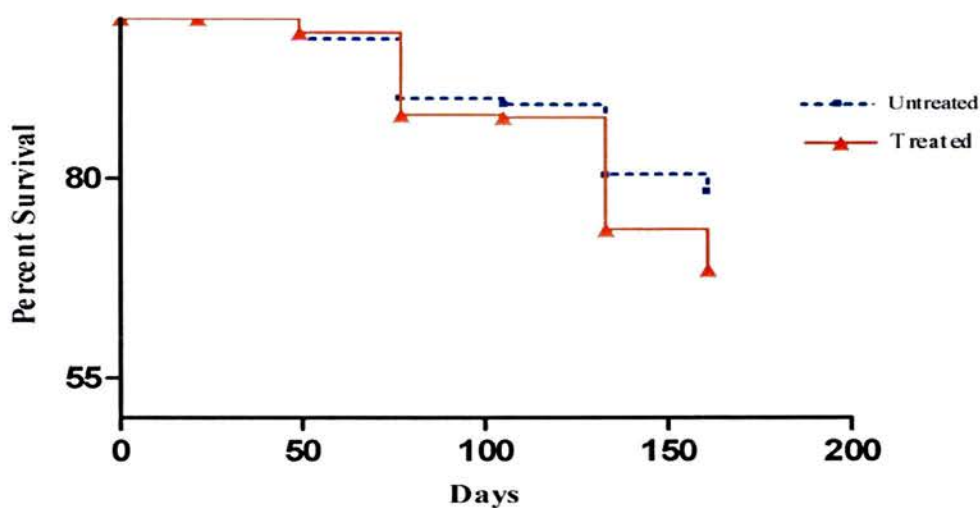


Figure 53: Kaplan-Meier Survival plot for *T. vivax* infections in ISMM treated and untreated cattle from the villages of Tororo district.

Table 36 Animals at risk of infection with *T. vivax* and survival proportion of cattle from Tororo villages.

Days	Animals at risk		Survival proportions	
	Treated	Untreated	Treated	Untreated
0	306	381	100	100
21	306	381	100	100
49	294	325	98	98
77	286	287	88	90
105	246	247	88	89
133	144	122	74	81
161	59	72	69	78

Comparative analyses of the survival curves of the *T. vivax* infection data from the treated and untreated animals over a period of 161 days in Busia show that there were a significant difference between the two groups, ( $\chi^2 = 13.8$ ,  $p = 0.0002$ ), Figure 54. The analysis shows that out of the initial 186 animals at risk in the treated group 165 were censored while out of the 141 animals at risk in the untreated group 105 were censored. The results also indicate that at the end of the study, 95 animals in the treated group were at risk compared to 52 in the untreated group. The results also show that 86% of the treated animals and 66% of the untreated group survived without infection up to 161 days, Table 37.

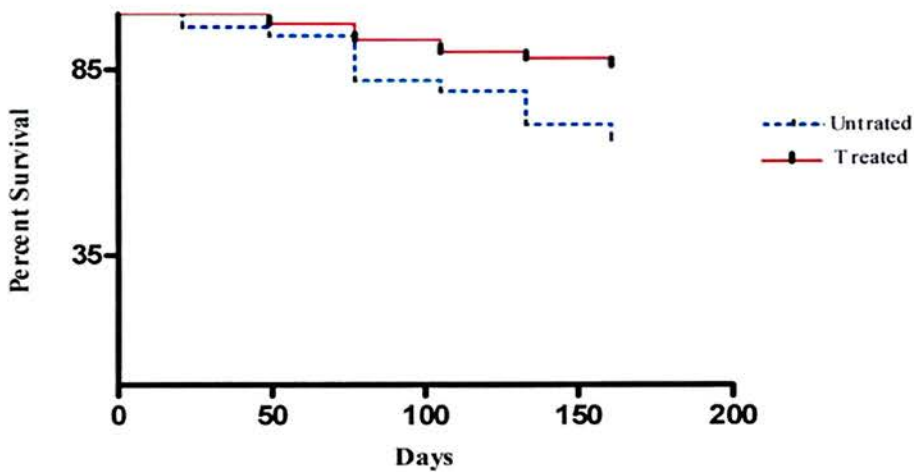


Figure 54: Kaplan-Meier Survival plot for *T. vivax* infections in ISMM treated and untreated cattle from the villages of Busia district.

Table 37: Animals at risk of infection with *T. vivax* and the survival proportion of cattle from Busia villages.

<u>Days</u>	<u>Animals at risk</u>		<u>Survival proportions</u>	
	<u>Treated</u>	<u>Untreated</u>	<u>Treated</u>	<u>Untreated</u>
0	186	141	100	100
21	186	141	100	96
49	183	126	97	94
77	162	109	93	82
105	137	83	90	79
133	108	71	88	70
161	95	52	86	66

Comparative analyses of the survival curves of the *T. vivax* infection data in both districts over a period of 161 days from the treated and untreated animals show that there was no significant difference between the two groups, , ( $\chi^2 = 0.95$ ,  $p = 0.33$ ), Figure 55. The analysis shows that out of the initial 430 animals at risk in the treated group 165 were censored while out of the 400 animals at risk in the untreated group 105 were censored. The results also indicate that at the end of the study, 95 animals in the treated group were at risk compared to 52 in the untreated group. The results also show that 74% of the treated animals and 70% of the untreated group survived without infection up 161 days, Table 38.

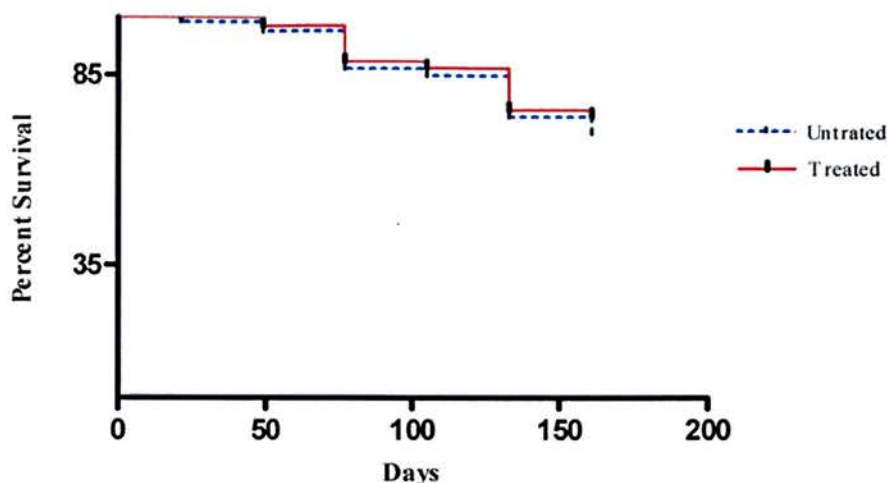


Figure 55: Kaplan-Meier Survival plot for *T. vivax* infections in ISMM treated and untreated cattle in both districts

Table 38 Animals at risk of infection with *T. vivax* and survival proportion of cattle from both districts.

Days	Animals at risk		Survival proportions	
	Treated	Untreated	Treated	Untreated
0	430	400	100	100
21	430	400	100	99
49	415	336	98	96
77	386	297	88	86
105	321	244	87	85
161	95	52	74	70

#### 6.3.4 The effect of isometamidium chloride (ISMM) treatment of cattle against all trypanosome infections in both districts

Comparative analyses of the survival curves of the infection with all trypanosome species in the treated and untreated animals from both districts over a period of 161 days show that there was a significant difference between the two groups, ( $\chi^2 = 5.14$ ,  $p = 0.0234$ ) (Figure

56). However, the analysis shows that 74% of the animals in both groups survived without infection with any of the three species of trypanosomes up to 161 days, Table 39.

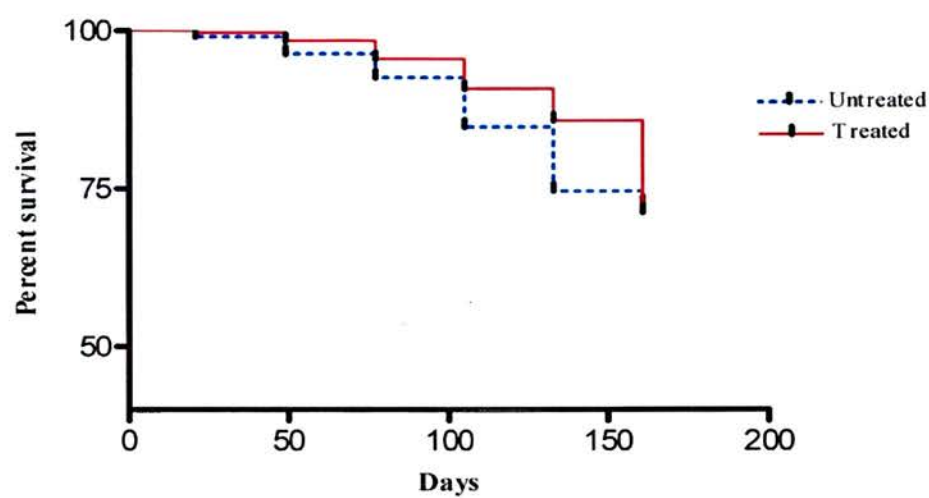


Figure 56: Kaplan-Meier Survival plot for all trypanosome species infections in ISMM treated and untreated cattle in both districts

Table 39 Animals at risk of infection with all trypanosome species in both districts and survival proportion of cattle from both districts.

Days	Animals at risk		Survival proportions	
	Treated	Untreated	Treated	Untreated
0	977	903	100	100
21	977	903	100	100
49	939	745	99	92
77	868	642	93	91
105	769	534	80	82
133	520	327	80	77
161	351	187	74	74

### 6.3.5 Time to trypanosome re-infection of cattle following isometamidium chloride prophylactic treatment

The time to re-infection of animals with *T. brucei* in the villages of Tororo and Busia following treatment with isometamidium chloride, is shown on Figure 57. The results show that the cumulative proportion of re-infection, (the proportion of animals that were reinfected over a period of six months), reached 32% after six months from the treatment date in the group of animals that had detectable trypanosomes prior to treatment compared to 36% in the animals that had no trypanosome the infection,  $\chi^2 = 0.06$ ,  $p = 0.8$  in the same period. In the latter group trypanosomes were detected as early as day 21 (2.5%) after treatment while in the group that were infected prior to treatment the trypanosomes were detected from day 77 (2.5%).

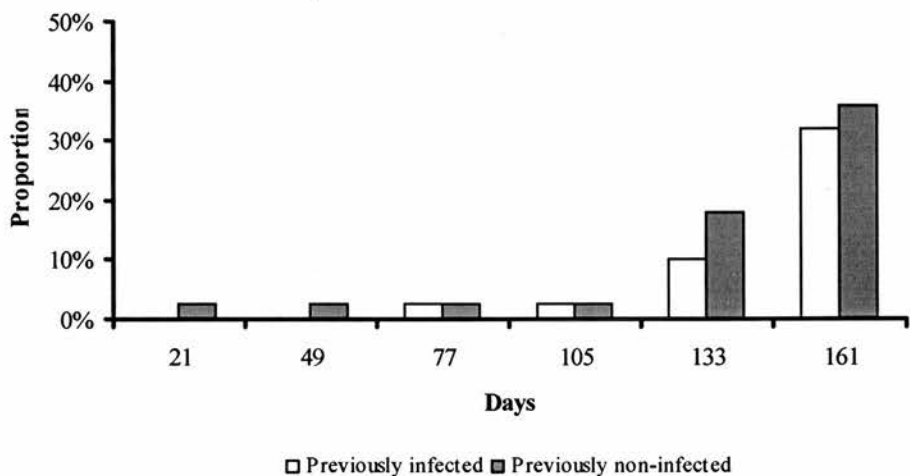


Figure 57: Proportion of animals re-infected with *T. brucei* in both districts

The time to re-infection of animals with *T. congolense* in the villages of Tororo and Busia is shown on Figure 58. The results show that the cumulative proportion of re-infection reached 21% after four months from the treatment date compared to 16% ( $\chi^2 = 0.8$ ,  $p = 0.4$ ), in the animals that had detectable trypanosomes prior to treatment and remained the same after six months. Re-infection was detected in the group that had had trypanosomes prior to treatment by day 49, (1.5%) while in the group that had no detectable trypanosomes the infection was detected by day 77 after treatment.



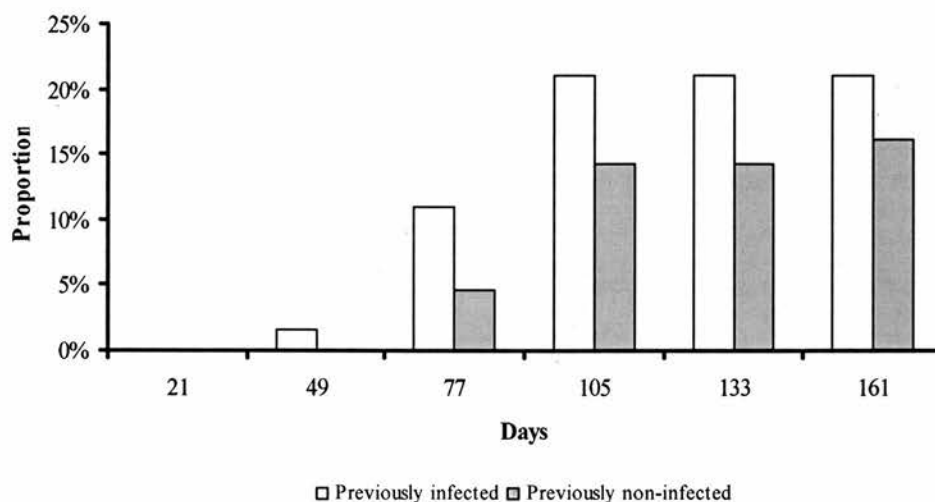


Figure 58: Proportion of animals re-infected with *T. congolense* in both districts

The time to re-infection of animals with *T. vivax* in the villages of Tororo and Busia is shown on Figure 59. The results show that the cumulative proportion of re-infection reached 36% in the group that had prior infection, after five months from the treatment date compared to 40% in the group that had no prior infection, ( $\chi^2 = 0.18$ ,  $p = 0.7$ ), in the corresponding period. Trypanosomes were detected in the group that had infection prior to treatment as early as day 21 after treatment and the proportion rose steadily up to the peak. In the group that had no detectable trypanosomes prior to treatment the infection was detected on day 77 after treatment and rose steadily thereafter up to the peak after six months.

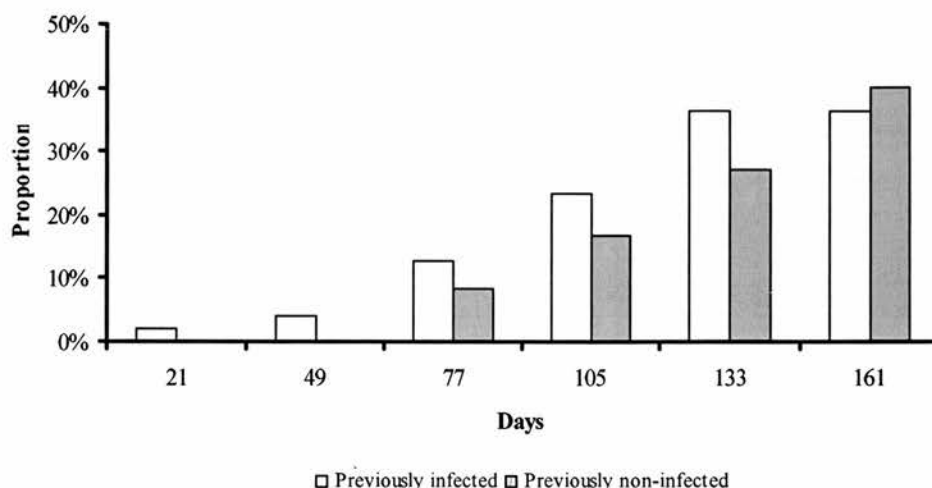


Figure 59: Proportion of animals re-infected with *T. vivax* in both districts

The time to re-infection of animals with all the three trypanosome species in the villages of Tororo and Busia is shown on Figure 60. The results show that the proportion of re-infection reached 29.86% after six months from the treatment date. The proportion of infection in the group that had no detectable trypanosomes prior to treatment reached 30.8% in the corresponding period. In both groups trypanosomes were detected as early as day 21 after treatment (<1%) but the cumulative proportion was higher in the group that had trypanosomes prior to treatment from day 49 post treatment.

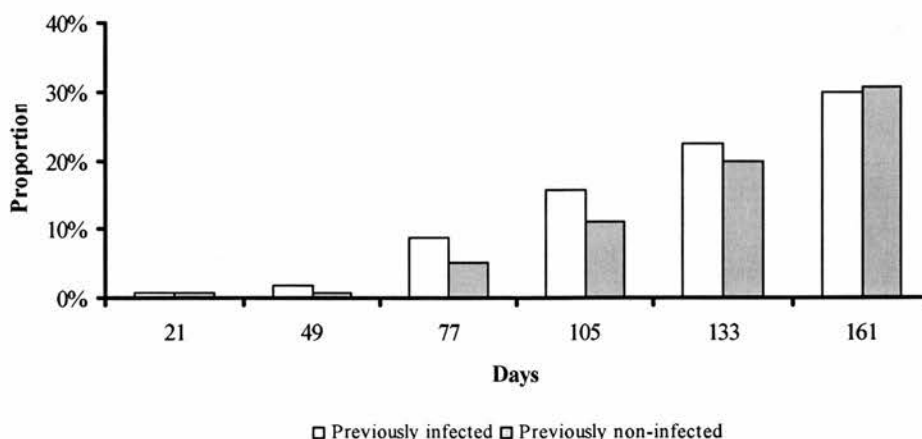


Figure 60: Proportion of animals re-infected with any of the three trypanosome species in both districts

**6.3.6 Analysis of diminazene treatment of cattle against trypanosomes**

The results of the analysis to test whether there was any effect on the change in prevalence of trypanosomes 28 days after treatment with diminazene, as determined by microscopic examination are shown in Figure 61. The proportion of positive observations on the following month after treatment was 6.88% (C.I. 4.56 – 10.06) in animals which were previously diagnosed with the parasites while those which were diagnosed negative for parasites were 4.12% (3.68 – 4.59). The proportions were significantly different,  $\chi^2$  6.3,  $p = 0.01$

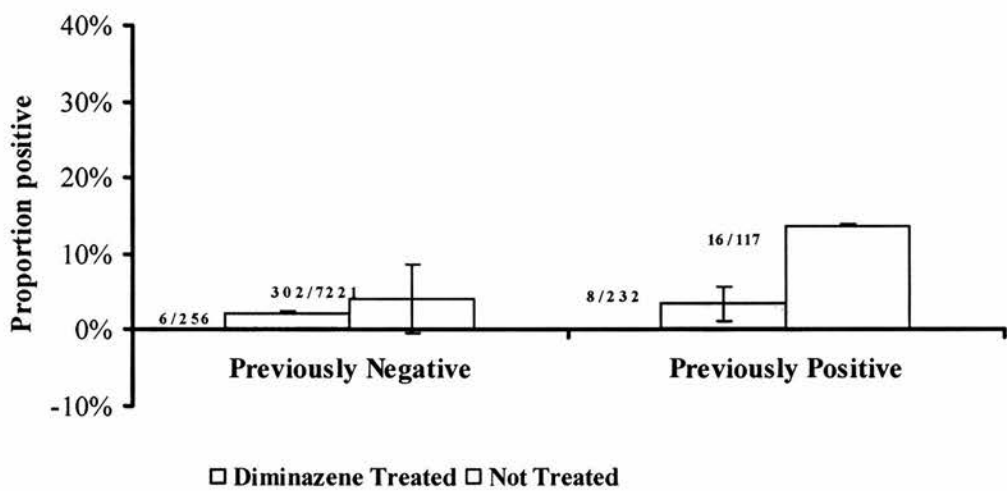


Figure 61: Proportion of animals with detectable trypanosomes by microscopy

Overall, the proportion of trypanosome positive observations in treated animals was 2.86% (1.57 – 4.75) while in the untreated animals it was 4.32% (3.88 – 4.82) with microscopic examination. The results indicate that there was no interaction between diminazene treatment and the proportion of animals with detectable trypanosomes by microscopy, ( $F_{1,6979} = 2.5504$  ;  $p = 0.11$ ). The relative risk (RR) was 33.8% more risk of positive diagnosis in untreated, regardless of whether they had been diagnosed with trypanosomes in the previous month or not.

The results of the analysis to test whether there was any effect on the change in prevalence of trypanosomes after 28 days as determined by PCR amplification are shown in Figure 62. The proportion of positive observations on the following month after treatment was 19.15% (16.5 – 22.0) in animals which were previously diagnosed with the parasites while those which were diagnosed negative for parasites were 13.09% (12.1 – 14.1).

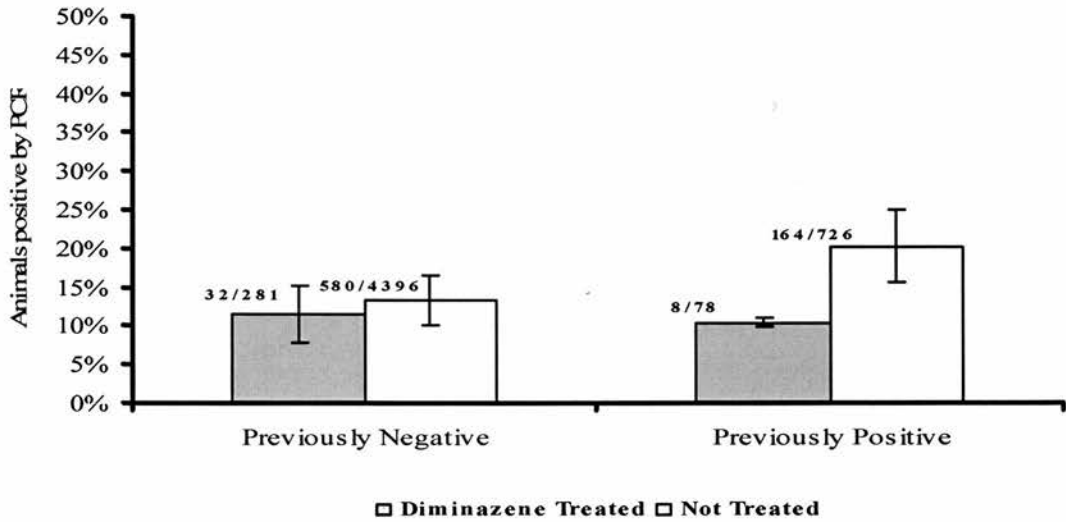


Figure 62: Proportion of animals with PCR detectable trypanosomes

Overall the proportion of trypanosome positive observations in treated animals was 11.14% (8.01 – 14.86) while in the untreated animals it was 14.17% (13.2 – 15.2), with PCR amplification. The results indicate that there was no interaction between diminazene treatment and the proportion of animals with detectable trypanosomes, ( $F_{1,4763} = 2.2152$  ;  $p = 0.1367$ ). The relative risk, (RR) was 18.93% more risk of positive diagnosis in untreated cattle regardless of whether they had been previously diagnosed with trypanosomes or not.

## 6.4 DISCUSSION

Isometamidium chloride has always been the drug of choice for the prophylactic treatment of cattle in many parts of trypanosome infested Africa. However, this drug along with homidium and the therapeutic diminazine aceturate have been in use in Africa for more than 40 years. Subsequently, there have been many reports of wide spread trypanosome resistance to these drugs, (Geerts, *et al*, 2001; El Rayah, *et al*, 1999; Geerts and Holmes, 1998; Shinyangwe, *et al*, 1999; Afewerk, *et al*, 2000; Mulugeta, *et al.*, 1997; Clausen, *et al*, 1992). Uganda is one of the countries where trypanosome drug resistance has been reported, (Mwambu and Mayende, 1971; Matovu, *et al*, 1997; Enyaru, *et al*, 1998). However, the reports in Uganda describe only a few isolates of human trypanosomes, therefore the extent of drug resistance for other trypanosome species in cattle in Uganda in general and South Eastern region in particular is unclear.

### 6.4.1 Effect of isometamidium chloride treatment of cattle against *T. brucei* infection

A comparison of the incidence of infections with *T. brucei* in both isometamidium treated and untreated cattle from Busia and Tororo districts by using survival analysis was done. The results showed that there was no significant difference in infection rate with *T. brucei* between treated and untreated cattle in Tororo district, Figure 47, ( $p = 0.074$ ), with 63% of the treated animals surviving without PCR detectable infections up to 161 days compared to 49% in the untreated group, Table 30. Similarly, there was no significant difference between the two groups in Busia district, Figure 48, ( $p = 0.118$ ), with 46% of the treated animals surviving without PCR detectable infection up to 161 days compared to 63% in the untreated animals, Table 31.

When all the data from both districts were pooled together the result showed no significant difference between the two groups, Figure 49, ( $p = 0.986$ ), with 22% of the treated animals surviving up to 161 days without PCR detectable infection, compared to 54% in the untreated group (Table 33). However, the results are contrary to what had been found previously that isometamidium chloride treated cattle had higher proportion of cattle free of

*T. brucei* re-infection than the untreated ones, (Magona, *et al.*, 2004). These results further confirm that treatment of cattle had a negative rather than a protective effect in the control of infection with *T. brucei* in cattle as had been shown in Chapter 6. It is not clear what this observation could be attributed to. However, a more likely explanation, as in the previous Chapter where the prevalence of *T. brucei* was found to have an upsurge in treated animals in the sixth month in both districts and again in the tenth month in Tororo and thirteenth month in Busia, is that it could have been due to the fact that the animals lost the acquired resistance to this species of trypanosome following treatment. The other possibility is that of drug resistance, although in a previous study, Enyaru and others, (1998), concluded from their *in vivo* experiments in mice with a strain of *T. brucei rhodesiense* that drug resistant trypanosomes are probably not circulating in SE Uganda. It is important to note that some of these *T. brucei* infections had the characteristic *T. b. rhodesiense* *SRA* gene, as has been shown in Chapter 6, and therefore are likely to be human infective, thus posing a potential danger to human health.

The cumulative proportion of re-infection of cattle with *T. brucei* following treatment reached 32% in the group of animals that had trypanosomes before treatment while the proportion reached 36% in the group that had no trypanosomes prior to treatment, Figure 57. These results suggest that the infection rate was the same in treated and untreated animals and could be attributed to new infections, rather than breakthrough due to treatment failure. However, the results also imply that the animals could only be protected for four months after which the animals become vulnerable to new and higher infections.

#### **6.4.2 Effect of isometamidium chloride treatment of cattle against *T. congolense* infection**

When the comparison was done of the incidence of infections with *T. congolense* in both isometamidium treated and untreated cattle from Busia and Tororo districts by using survival analysis, the results showed variations in the two districts. In Tororo the difference in infection between treated and untreated animals was statistically highly significant, Figure 50, ( $p = 0.0001$ ) although the proportion of the animals that survived without PCR



detectable infections up to six months was 96% in the treated and 88% in the untreated groups respectively, Table 33. However, there was no significant difference in infection rate with *T. congolense* between treated and untreated cattle in Busia district, Figure 51, ( $p = 0.808$ ), with 74% of the animals surviving without PCR detectable infection up to 161 days in both groups, Table 34. This would be consistent with a previous study where it was shown that the proportion of untreated animals remaining free of infection was also high, (Magona, *et al.*, 2004).

The cumulative proportion of re-infection of cattle with *T. congolense* following treatment reached 21% in the group of animals that had trypanosomes before treatment while the proportion reached 16% in the group that had no trypanosomes prior to treatment. The proportions were however, not significantly different and therefore the results suggest that there was no protection in the animals that had treatment. The slight difference in proportions observed could imply that some of the infections detected in the animals that had prior infections before treatment could be attributed to breakthroughs due to treatment failure as well as new infections. However, the results also imply that the animals could only be protected for four months after which the animals became vulnerable to new infections, Figure 58.

When all the data from both districts were pooled together the results showed that there was significant difference between the two groups, Figure 52, ( $p = 0.0002$ ), with 89% of the treated animals surviving up to six months without infection compared to 83% in the untreated group, Table 35. These results suggest that treatment of cattle had an overall protective effect in the control of infection with *T. congolense* in cattle for six months and are consistent with the finding that the proportion of both isometamidium and diminazene treated animals remaining free of infection was higher than that of untreated animals, (Magona, *et al.*, 2004). The results from the two districts are quite different and appear to be contradictory. However, it should be recalled that the data on re-infection of cattle from Tororo with *T. congolense* showed that treatment conferred some protection which was otherwise masked by the data from Busia that did not show any protection. It should also be

recalled that the prevalence of *T. congolense* was higher in Busia cattle than in Tororo, resulting in the overall no protection net effect.

#### **6.4.3 Effect of isometamidium chloride treatment of cattle against *T. vivax* infection**

When the comparison was done of the incidence of infections with *T. vivax* in both isometamidium treated and untreated cattle from Busia and Tororo districts by using survival analysis, the results also showed variations in the two districts. In Tororo the difference in infection between treated and untreated animals was not significant, Figure 53, ( $p = 0.12$ ), while the proportion of the animals that survived without infection up to six months was 69% in the treated and 78% in the untreated groups respectively, Table 36, suggesting that treatment had an adverse effect rather than protecting the animals against *T. vivax* infection in Tororo. However, this could be attributed to the high prevalence of the trypanosome species observed in the area (Figure 42) since there was significant difference in infection with *T. vivax* between treated and untreated cattle in Busia district, Figure 54, ( $p = 0.0002$ ), where the prevalence was low. In Busia, the animals that survived without infection up to six months in the treated group were 86% compared to 66% in the untreated group (Table 37) suggesting that treatment protected the animals from infection. This is consistent with the finding of Magona, *et al.* (2004). It should be noted that the studies by Magona and others (2004) were conducted in areas that were under suppressed tsetse population and therefore lower prevalence of trypanosomiasis in cattle. As with *T. congolense*, the results appear to be contradictory but this could be due to differences in prevalence.

The cumulative proportion of re-infection of cattle with *T. vivax* following treatment reached 36% in the group of animals that had trypanosomes prior to treatment while the proportion reached 40% in the group that had no trypanosomes, Figure 59. Generally, the proportion of animals with infection was higher in the group that had trypanosomes prior to treatment than those that did not have infection. These results suggest that the infections could have been both breakthroughs due to treatment failure and new infections, since

trypanosomes could be detected from the group that had infection prior to treatment 21 days after treatment (2%) while in the animals that did not have infection prior to treatment trypanosomes were first detected from day 77, (8%). However, the results also imply that treatment of the animals could only confer protection from new infections for three months. These observations are consistent with several studies that have shown wide-spread isometamidium chloride treatment failure and lack of protection against trypanosome infection even when cattle are examined by microscopy, (Clausen, *et al.*, 1992; Geerts and Holmes, 1998; McDermott, *et al.*, 2003) and especially by PCR amplification on field samples, (Gall, *et al.*, 2004).

When all the data from both districts were pooled together the result showed that there was significant difference between the two groups, Figure 55, ( $p = 0.0002$ ). The results also show that 74% of the treated animals survived up to six months without infection compared to 70% in the untreated group, Table 38. These results imply that treatment of cattle had an overall protective effect in the control of infection with *T. vivax* in cattle for six months. The results suggest that *T. vivax* infection could be controlled with drug treatment in areas where the prevalence was lower but not in Tororo where it was high right from the baseline, as was observed in Chapters 5 and 6 for the prevalence of *T. vivax* in both districts, Figure 42.

#### **6.4.4 Effect of isometamidium chloride treatment of cattle against all trypanosome species infection**

When the data for all trypanosome species infection was combined and the comparison done in both isometamidium treated and untreated cattle from Busia and Tororo districts the results showed that there was significant difference, ( $p = 0.0234$ ), in infection between treated and untreated animals, Figure 56, although the proportion of the animals that eventually survived without infection up to six months was 74% in both groups, Table 40. The cumulative proportion of re-infection of cattle with any trypanosome species following treatment reached 30% in the group of animals that had trypanosomes before treatment while the proportion reached 31% in the group that had no trypanosomes prior to treatment

in six months, Figure 60. The results also show that there were detectable trypanosomes in both groups as early as day 21, (<1%) but reached above 10% by day 105 after treatment. These results suggest that the infections could have been both breakthroughs due to treatment failure and new infections. However, the results also imply that treatment of the animals could only confer protection against infection with any species of trypanosomes for three months.

Overall, the results showed that significant differences in infection rate due to drug treatment occurred only in the areas where the prevalence was low, for example, in Tororo where the prevalence of *T. congolense* was low or in Busia where the prevalence of *T. vivax* was low. This seems to suggest that effectiveness of prophylactic treatment of cattle against animal trypanosomiasis could be linked to the disease transmission rate and can be achieved where trypanosome challenge is low. It should be noted that animals with low challenge below the detection threshold of microscopy show no clinical signs of the disease and may continue to be as productive as the ones without infection. Indeed it has been shown that where drug resistance has been demonstrated prophylactic treatment as a control method still had some benefits to cattle, especially when combined with other methods such as vector control, (Rowlands, 1994). The results indicate that whereas it is possible to achieve some control of animal trypanosomiasis with isometamidium chloride treatment for up to six months in areas of low challenge it does not appear to have any effect in controlling *T. brucei* infections.

The results seem to suggest that there is a likelihood of drug resistant trypanosome species circulating in cattle from the two districts, contrary to the finding by Olila and others, (2002), in which they concluded that the trypanosome isolates from Mukono County, another area in SE Uganda, were all trypanocide sensitive. The possible explanation is that drug treatment does not clear infection but only reduces it to levels that cannot be detected by the conventional microscopic examination used in Olila's studies. Alternatively, it is possible that the high infections simply overwhelm the effect of prophylactic drug treatment.

#### 6.4.5 The effect of treatment of cattle with diminazene on trypanosome infection

When the analysis was performed to test whether there was any effect on the change in prevalence of trypanosomes 28 days after treatment with diminazene, as determined by microscopic examination, Figure 61, the proportion of positive observations in animals which were previously diagnosed with the parasites on the following month after treatment was found to be 6.88% (24/349) while those which were diagnosed negative for parasites one month prior to treatment was 4.12% (308/7479). Overall, the proportion of trypanosome positive observations in the untreated animals was a little more than twice, 4.32% (318/7338), the proportion of the treated animals, 2.86% (14/490), suggesting that treatment with diminazene led to a drastic decline of *T. brucei* in animals. This is consistent with a previous finding by Rushigajik, *et al.*, (1986), where it was observed that treatment with 7mg/kg body weight caused a drastic reduction. It should be noted that in this study the animals were given half the dose used in the previous study. The calculated relative risk (RR) indicated that there was 33.8% more risk of positive diagnosis in untreated animals, regardless of whether they had been diagnosed with trypanosomes in the previous month or not. However, the analysis indicated that there was no interaction between diminazene treatment and the detection of trypanosomes after 28 days by microscopy, ( $F_{1,6979} = 2.5504$ ;  $p = 0.1103$ ).

The analysis performed on the data obtained by PCR amplification, Figure 60, showed that the proportion of positive observations on the following month after treatment was 19.15% (154/804) in animals which were previously diagnosed with the parasites while those which were diagnosed negative for parasites were 13.09% (612/4677). Overall, as with microscopy, there was no major difference between proportion of trypanosome positive observations in treated animals, 11.41% (40/359), and the untreated animals, 14.07% (726/5122), with PCR amplification. The relative risk, (RR) indicated that there was 18.93% more risk of positive diagnosis in untreated animals regardless of whether they had been previously diagnosed with trypanosomes or not. This was about half of what was found with microscopy data, further indicating that there is more likelihood of detecting

trypanosomes in cattle by PCR than microscopy as had been found in Chapter 4. As with microscopy however, the analysis indicated that there was no interaction between diminazene treatment of cattle and trypanosome detection after 28 days, ( $F_{1,4763} = 2.2152$  ;  $p = 0.1367$ ).

Equally interesting was the fact that infected cattle had 74.78% less relative risk of trypanosome detection by microscopy, when compared with 44.39% in those which did not receive the drug while previously negative, Figure 61. The observation was more evident with PCR amplification method, 49% less relative risk in infected cattle compared to 13.69% for those which received drug while previously negative, Figure 62. These observations imply that diminazene treatment appeared to have some residual effect on trypanosome infection after 28 days. Since the effects of diminazene appears to be far more obvious when considering microscopy data which has poor diagnostic sensitivity, it is reasonable to conclude that diminazene lowers parasitemia rather than achieving complete clearance of the infection. This observation confirms the initial hypothesis advanced in this Chapter. It is worth noting that not all animals which received diminazene treatment had the infection cleared as determined by either technique, indicating that treatment of cattle with this drug cannot be relied upon to reduce the transmission of the parasite.



## **CHAPTER SEVEN**

### **7 ANALYSES OF THE INTERACTIVE FACTORS ASSOCIATED WITH TRYPANOSOME INFECTION IN CATTLE**

#### **7.1 INTRODUCTION**

The distribution and prevalence of various trypanosome species in cattle can be presented in proportionate formats as in Chapters 5 and 6. However, this format does not always include detailed information distinguishing the role of associating factors such as age, sex or combinations of these factors or associations between the prevalence and clinical factors such as anaemia. Equally important is the associations among different parasite species infecting the animals.

The use of statistical analyses on parasitological data can provide powerful means for understanding the biological processes involving parasite infections, such as the likelihood of exposure, the susceptibility of same or variable hosts or even the virulence and infectivity of the various parasite strains or species. However, powerful and reliable analysis of such data from natural infections is usually difficult and complicated where multiple samples are taken from same animals repeatedly, such as in longitudinal study carried out in this project, in which the data was collected from animals reared in several different locations under mixed farming management system with unpredictable natural infections in animals and where the distribution of parasites between hosts is likely to be aggregated. The observations from such studies are normally correlated temporally, through time or spatially. However, since these observations tend to bear some relationship with each other, it is difficult to describe the distribution of the parasites in the animals while taking into account the underlying associations.

Previous studies have recommended the use of mixed models in analyzing parasitological data, (Paterson and Lello, 2003). Such models can incorporate random effects such as anaemia, sex, age, or multiple infections with different species of parasites and / or treatments between groups of observations. Similar models have been used in studying the

susceptibility of trypanotolerant West African dwarf goats and trypanosusceptible breeds under challenge with *T. congolense*, (Faye, *et al.*, 2002) and the transmission of mixed *T.b. brucei*/*T. congolense* by tsetse fly, (Van den Bossche, *et al.*, 2004).

### **7.1.1 Objectives of the study in this Chapter**

The objectives of the analyses were to determine the relationship between the prevalence of various trypanosome species and the sex and age of the animals by using the data obtained by PCR amplification screening. This was achieved by using a generalized linear model that incorporated the terms: sex, age and sex/age of the animals with the prevalence of trypanosome species in cattle.

## **7.2 MATERIALS AND METHODS**

The Zebu cattle from both Busia and Tororo districts were categorized into males and females as well as into three age groups, 1-12 months, 13-24 months and above 24 months old. The ages of the animals were determined by asking the cattle owners in the villages at the start of the study, either the date of birth or how old the animals were believed to be as had been described in Chapter 2. Trypanosome infection data was collected from screening buffy-coat samples from cattle collected on FTA filter cards, (Whatmann Biosciences), by PCR amplification as detailed in Chapter 3. Data from PCR amplification of samples collected over a period of 16 months was used in these analyses, since it had been established that the method has high specificity and sensitivity in detecting the various trypanosome species. The method also provided substantial sample size that could be analyzed. The data obtained by microscopy was not adequate for analysis.

### **7.2.1 Analysis of the interaction of trypanosome infections with age, sex or age and sex**

The animals were grouped according to their sexes and then categorized into three age groups, namely, calves (1-12 months old), young animals (13-24 months) and adults (animals over 24 months old). The interaction of the prevalence of *T. brucei*, *T. congolense*

and *T. vivax* as determined by PCR amplification in cattle with age and sex was computed using the following linear mixed models:

- *T. brucei* ~ age\*sex on n (number of observations) = 6131 on 797 animals.
- *T. congolense* ~ age\*sex on n (number of observations) = 3576 on 822 animals.
- *T. vivax* ~ age\*sex on n (number of observations) = 3576 observations on 822 animals.

These models could be simplified to allow for further reduction of terms in order to determine the association of individual factors with various trypanosome species separately and the analyses were done using the R-software version 2.1.1, (2005, The R-Development Core Team, ([www.r-project.org](http://www.r-project.org))).

### 7.3 RESULTS

#### 7.3.1 Interaction between the prevalence of *T. brucei* and sex, age or a combination of age and sex

The results show that across individual village level *T. brucei* could be detected in more animals that were 13 months old and above than in the age group below 12 months. The prevalence was highest in the animals over 25 months old, Figure 63.

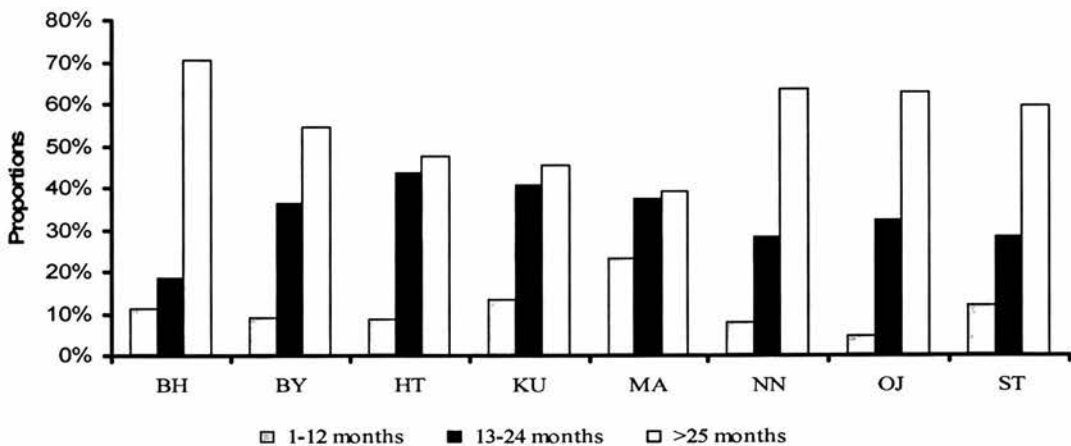


Figure 63: Detection of *T. brucei* in animals of different age groups in different villages. (BH - Bunghanji, BY - Buyimini, HT - Hitunga, KU - Kubo, MA - Magoje, NN - Nanjeho, OJ - Ojilai, ST - Sitengo villages).

When the data from all the villages were pooled together and analyzed, the results showed that there was no interaction between the prevalence of *T. brucei* in cattle and their age and sex combined ( $F_{2\ 5330} = 0.0279$ ,  $p = 0.972$ ). When the model was simplified by removing the age in order to determine the association with sex alone, the result showed that there was still no interaction between the prevalence and sex of the animals,  $F_{1\ 795} = 0.5729$ ,  $p = 0.4493$ , although there was some variation in the proportions (26% males vs. 47% females) as the animals grow older, Figure 64.

However, when the model was simplified by removing sex in order to determine the association with age, the simplified model:  $T. brucei \sim age$ , it was observed that the animals in the 13 months and above age group were more likely to be *T. brucei* detectable than those less than 12 months old, ( $F_{2\ 5332} = 9.4243$ ,  $p = 0.0001$ ). Since the proportion of *T. brucei* detectable animals within the 13-24 months group was almost the same as that in the adults group, 10.40% compared to 9.97%, Table 40, they were subsequently grouped together, resulting in a mean proportion of 10.14% with a 95% exact binomial CI = 9.32 and 11.00, for adults compared to the proportion of 6.37% in calves with a 95% exact binomial CI = 4.95 and 8.05, Figure 64. The relative risk (RR) of *T. brucei* PCR detectable calves compared with 62.88%, in the adults, thus the adults were approximately 37% more likely to be *T. brucei* detectable by PCR amplification than those below 13 months.

Table 40: Distribution of animals by age with PCR detectable *T.brucei*.

	<u>1-12 months</u>	<u>13-24 months</u>	<u>&gt;24 months</u>
PCR not detectable	955	1723	2870
PCR detectable	65	200	318
Total	1020	1923	3188
Proportion of detectable	6.37%	10.40%	9.97%

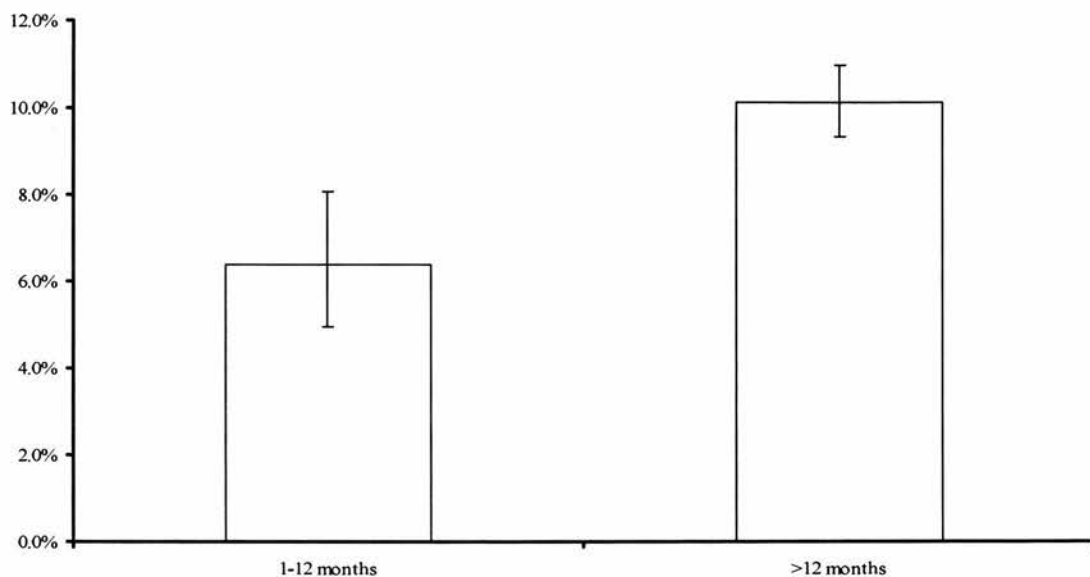


Figure 64: Proportions of animals with PCR detectable *T. brucei* in each age group.

### 7.3.2 Interaction between the prevalence of *T. congolense* and age, sex or age plus sex

The results show that except for Hitunga village (HT), across individual village level *T. congolense* could be detected in more animals that were 13 months old and above than in the age group below 12 months, Figure 65.

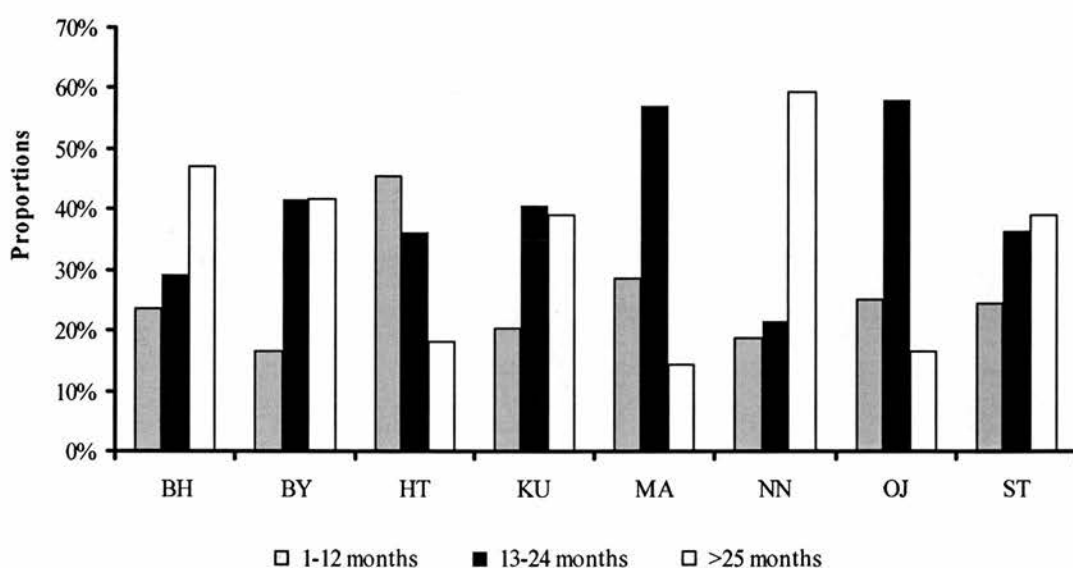


Figure 65: Detection of *T. congolense* in animals of different age groups in different villages. (BH - Bunghanji, BY - Buyimini, HT - Hitunga, KU - Kubo, MA - Magoje, NN - Nanjeho, OJ - Ojilai, ST - Sitengo villages).

When the data from all the villages were pooled together, it was observed that there was no interaction between prevalence of *T. congolense* in the animals and their age and sex combined, it was found that there was no interaction,  $F_{2, 2750} = 2.5063$ ,  $p = 0.8182$ . When the model was simplified by removing the age in order to determine the association with sex alone, it was also observed that there was no interaction between the prevalence and sex of the animals,  $F_{1, 820} = 0.0139$ ,  $p = 0.9063$ , although there was some variation in the proportions, (10% males vs. 17% females), as the animals grow older. However, when the analysis was done to determine the association between prevalence and age on a simplified model:  $[T. congolense \sim \text{age}]$ , in which sex was omitted, it was observed that the animals in the 1-24 months age group were more likely to have *T. congolense* detectable by PCR amplification, than the adults,  $F_{2, 2752} = 20.4843$ ,  $p < 0.0001$ . Since the proportion of *T. congolense* detectable animals within the 1-12 months age group was almost the same as that in 13-24 months (7.10% vs. 7.41%), Table 41, they were subsequently grouped together, resulting in a mean proportion of 7.28% with 95% exact binomial CI = 6.15% and



8.56%, for animals up to 24 months old compared with 4.85% with 95% exact binomial CI = 3.88% and 5.98% for the adults, Figure 84. The relative risk (RR) of *T. congolense* detectable by PCR in the 1-24 months age group compared with 66.63% in the adults, thus the animals in the age group between 1 and 24 months were 33% more likely to be *T. congolense* detectable by PCR amplification than those above 24 months.

Table 41 Distribution of animals by age with PCR detectable *T.congolense*

	<u>1-12 months</u>	<u>13-24 months</u>	<u>&gt;24 months</u>
PCR not detectable	694	1037	1627
PCR detectable	53	83	83
Total	747	1120	1710
Proportion of detectable	7.10%	7.41%	4.85%

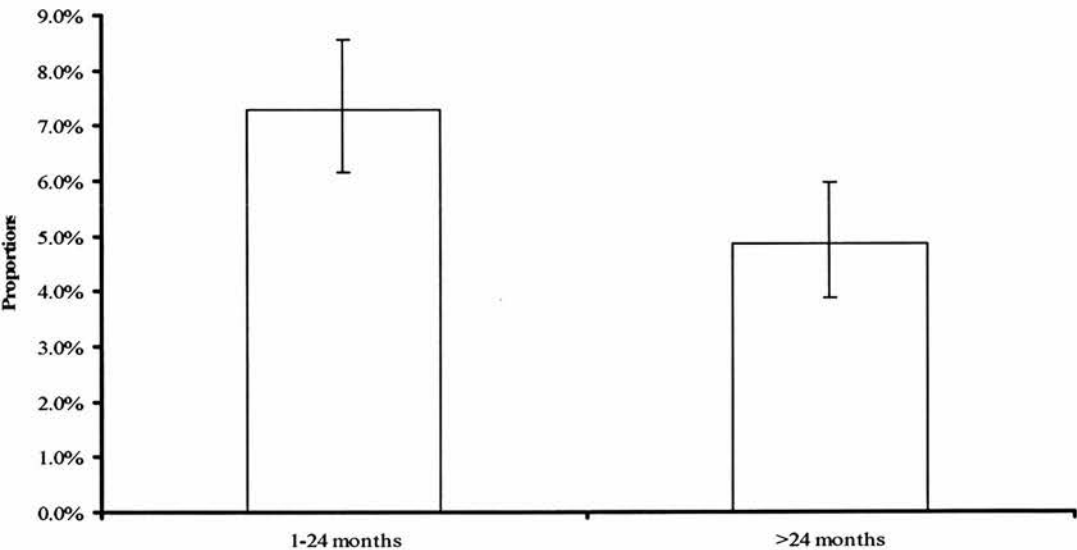


Figure 66: Proportions of animals with PCR detectable *T. congolense* in each age group.

**7.3.3 Interaction between the prevalence of *T. vivax* and age, sex and age and sex**

The results show that except for Bunghaji village (BH), across individual village level there were more *T. vivax* detectable animals by PCR amplification in the 1-24 months age groups than adults across the villages, Figure 67.

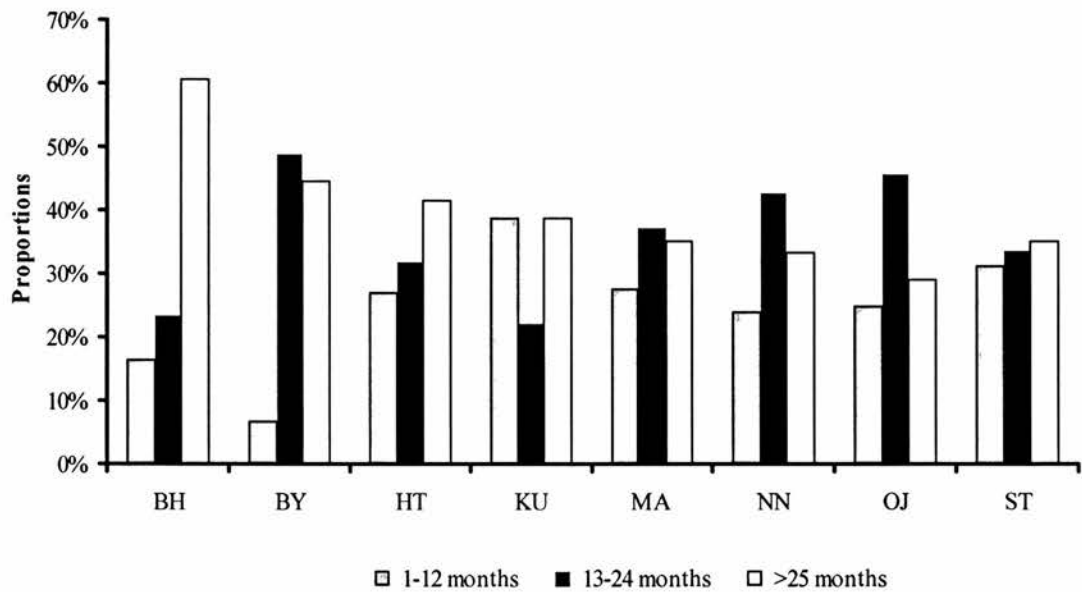


Figure 67: Detection of *T. vivax* in animals of different age groups in different villages. (BH - Bunghanji, BY - Buyimini, HT - Hitunga, KU - Kubo, MA - Magoje, NN - Nanjeho, OJ - Ojilal, ST - Sitengo villages).

When the data from all the villages were pooled together, it was observed that there was no interaction between the prevalence of *T. vivax* in the animals and their age and sex when combined,  $F_{2\ 2825} = 0.9675$ ,  $p = 0.3801$ . When the model was simplified by removing age in order to determine the association between prevalence and sex alone it was also observed that there was no interaction between the prevalence and the sex of the animals,  $F_{1\ 825} = 2.0449$ ,  $p = 0.1531$ , although there was some variation in the proportions, 15% males compared to 25% females, as the age of the animals increased. However, when the analysis was done on a simplified model where age alone was considered:  $T. vivax \sim age$  in which sex was removed, the analysis showed that the animals in the 1-24 months age group were

more likely to have *T.vivax* detectable animals by PCR amplification, than the adults,  $F_{2, 2827} = 7.4177$ ,  $p = 0.0006$ . Since the proportion of *T. vivax* detectable animals within the 1-12 months group was almost the same as that in the 13-24 months age group, (11.19% vs. 10.44%), Table 42, they were subsequently grouped together, resulting in a mean proportion of 10.74% with 95% exact binomial CI = 9.38% and 12.23%, for animals up to 24 months old compared with 7.65% with 95% exact binomial CI = 6.46% and 8.99%, Figure 68. The relative risk (RR) of *T.vivax* detection by PCR in calves compared with adults was computed to be 71.27%, thus the animals in the age group between 1 and 24 months were approximately 29% more likely to be *T.vivax* detectable by PCR amplification than those above 24 months.

Table 42: Distribution of animals by age with PCR detectable *T. vivax*

	<u>1-12 months</u>	<u>13-24 months</u>	<u>&gt;24 months</u>
PCR not detectable	667	1012	1641
PCR detectable	84	118	136
Total	751	1130	1777
Proportion of PCR detectable	11.19%	10.44%	7.65%

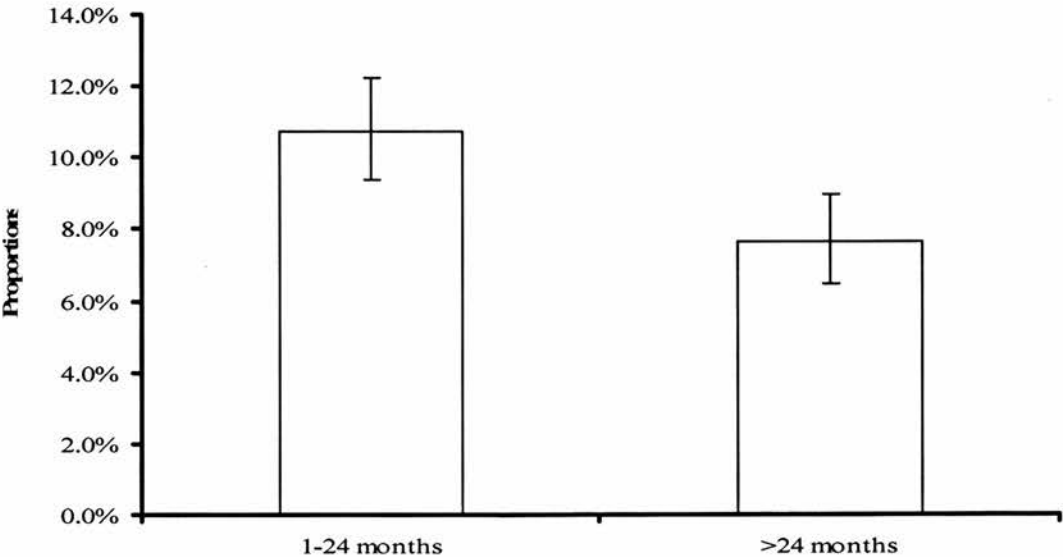


Figure 68: Proportions of animals by age with PCR detectable *T. vivax* group.

7.3.4 The prevalence of all trypanosome species in various animal age-groups

In an average of 4,454 observations on about 815 animals the distribution of the three pathogenic trypanosome species in various animal age-groups show that *T. congolense* and *T. vivax* were detected by PCR in more 1-12 months age group, 25% and 24% than *T. brucei*, 11%. In the animals within 12-24 months age group PCR detection levels were almost similar at 34% for *T. brucei*, 38% for *T. congolense* and 35% for *T. vivax*. However, in the animals that were older than 24 months, more animals were *T. brucei* detectable at 55%, followed by *T. vivax* at 40% and *T. congolense* at 38%, Table 44, Figure 69.

Table 43 Number of animals PCR detectable in different age-groups that are with various trypanosome species

Age-groups (months)	Number of animals		
	<i>T. brucei</i>	<i>T. congolense</i>	<i>T. vivax</i>
1-12	65(11.1%)	53(24.2%)	84(24.8%)
13-24	200(34.3%)	83(37.9%)	118(34.8%)
>24	318(54.5%)	83(37.9%)	136(40.1%)
Total	583	219	339

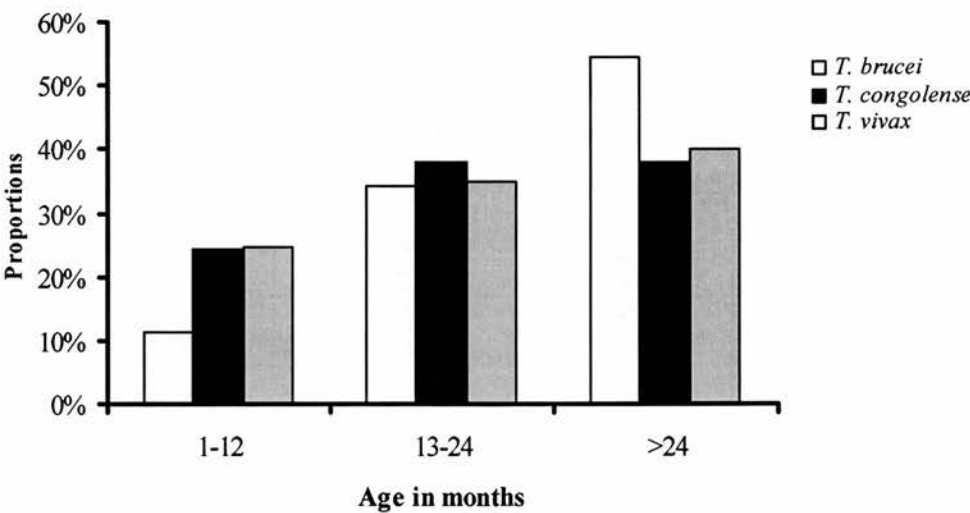


Figure 69: Distribution of trypanosome species detectable by PCR in animals by age

## 7.4 DISCUSSION

Several factors including sex, age and a combination of both were tested in a mixed effect model to determine their association with the prevalence of individual trypanosome species as well as all trypanosome species in cattle. The analysis revealed that the prevalence of all the trypanosome species had no association with the combination of the sex of the animals and their age,  $p > 0.5$ . In a simplified model in which the sex of the animals was considered independently, the analysis also showed that there was no association between the prevalence of all trypanosome species and sex,  $p = 0.449$ , although there was some variation in the proportions of animals detected with infection of *T. brucei*, 26% males compared to 47% females; for *T. congolense*, 10% males compared to 17% females and with *T. vivax*, 15% males compared to 25% females as the animals grow older. The analysis suggests that despite the differences in the proportions both sexes get infection equally. The differences in proportions between sexes could be attributed to the varied samples sizes as there were more females in the study than males.

However, in a simplified model that considered the age of the animals alone, the analysis clearly revealed significant difference,  $p = 0.0001$ , as it showed that *T. brucei* could be detected more frequently in samples from animals that were 13 months old and above than in calves below 12 months old. The prevalence was highest in adult animals over 24 months old, Figure 64, suggesting that *T. brucei* infections increased with the age of the animals. When the animals infected with *T. brucei* in the 13-24 months group were subsequently grouped together, the resulting mean proportion of infected cattle was 10.14% for adults compared to 6.37% in calves, Table 40. The analysis showed that the relative risk of *T. brucei* PCR detectable calves compared to adults was 62.88%, indicating that the adults were approximately 37% more likely to have *T. brucei* infection as determined by PCR amplification than those below 13 months. The possible explanation for this observation is the fact that *T. brucei* is known to cause sub-clinical disease in cattle, (Morrison, *et al.*, 1983), therefore it is possible for infected calves to live and grow up with infection into adulthood, without showing any clinical signs that would warrant intervention by the farmer.

The simplified model also showed the association between prevalence and age, the prevalence of *T. congolense*. However, the analysis showed that the animals in the 1-24 months age group were more likely to have *T. congolense* detectable by PCR amplification, than the adults,  $F_{2, 2752} = 20.4843$ ,  $p < 0.0001$ . Since the proportion of *T. congolense* detectable animals within the 1-12 months age group was almost the same as that in 13-24 months (7.10% compared to 7.41%), Table 41, they were subsequently grouped together, resulting in a mean proportion of 7.28% with 95% exact binomial CI = 6.15% and 8.56%, for animals up to 24 months old compared with 4.85% with 95% exact binomial CI = 3.88% and 5.98% for the adults, Figure 66. The relative risk (RR) of *T. congolense* detectable by PCR in the 1-24 months age group compared with 66.63% in the adults, thus the animals in the age group between 1 and 24 months were 33% more likely to be *T. congolense* detectable by PCR amplification than those above 24 months.

The results show that except for Bunghaji village (BH), across individual village level there were more *T. vivax* detectable animals by PCR amplification in the 1-24 months age groups than adults across the villages, Figure 67. As with *T. congolense*, when the analysis was done on a simplified model where age alone was considered:  $T. vivax \sim \text{age}$  it was observed that the animals in the 1-24 months age group were more likely to have *T. vivax* detectable animals by PCR amplification, than the adults,  $F_{2, 2827} = 7.4177$ ,  $p = 0.0006$ . Since the proportion of *T. vivax* detectable animals within the 1-12 months group was almost the same as that in the 13-24 months age group, (11.19% vs. 10.44%), Table 44, they were subsequently grouped together, resulting in a mean proportion of 10.74% with 95% exact binomial CI = 9.38% and 12.23%, for animals up to 24 months old compared with 7.65% with 95% exact binomial CI = 6.46% and 8.99%, Figure 68. The relative risk (RR) of *T. vivax* detection by PCR in calves compared with adults was computed to be 71.27%, thus the animals in the age group between 1 and 24 months were approximately 29% more likely to be *T. vivax* detectable by PCR amplification than those above 24 months. As with *T. congolense*, the possible explanation for this observation is the fact that *T. vivax* also causes a clinical disease that would necessitate intervention thereby reducing the prevalence in older animals.



These results are contrary to what had been observed in a study in the Ghibe Valley, southwest of Ethiopia where it was found that the prevalence of *T. congolense* increased with the age of the animals, (Rowlands, *et al.*, 1993). The possible explanation for this observation is that infections with *T. congolense* are much lower in parasitaemia in calves and therefore more difficult to detect with microscopic examination than with PCR amplification.

In an average of 4,454 observations on about 815 animals the distribution of the three pathogenic trypanosome species in various animal age-groups show that *T. congolense* and *T. vivax* were detected by PCR in more 1-12 months age group, 25% and 24% than *T. brucei*, 11%. The difference between the proportions of *T. congolense* and *T. vivax* on one hand and *T. brucei* in young animals was highly significant,  $\chi^2 = 62$ ,  $p < 0.0001$ . In the animals within 12-24 months age group PCR detection levels were almost similar at 34% for *T. brucei*, 38% for *T. congolense* and 35% for *T. vivax*. However, in the animals that were older than 24 months, more animals were *T. brucei* detectable at 55%, followed by *T. vivax* at 40% and *T. congolense* at 38%, Table 43, Figure 69. As has been mentioned above, the higher detectable *T. brucei* infections in adult animals could be due to the fact that it does not cause clinical disease in cattle. However, this needs further investigation in a controlled experiment where trypanosome transmission levels are also monitored.

## CHAPTER EIGHT

### 8 GENERAL DISCUSSION

#### 8.1 VECTOR BORNE DISEASES

Vector borne diseases cost Africa well over US\$ 4 billion per year in livestock production and other associated losses. Tsetse transmitted trypanosomiasis has been estimated to cost US\$ 4.5 billion yearly while tick-borne diseases such as theileriosis costs losses to the tune of US\$ 168 million per year, (Mukhebi, *et al.*, 1992), as they affect both human and livestock. Control of these diseases, which was handled relatively well during the pre-independence period, has largely ground to a halt in many countries due to social, economic and political problems that have plagued most of the affected countries. Further more, the current governments have not been able to put in place adequate control programmes.

Currently, control of these diseases depends mostly on vector control through the use of insecticides and other chemotherapeutic methods targeting the disease causing pathogens. Such control measures have become either too expensive or unreliable and there is therefore need to optimize them for maximum returns while minimizing their side effects. Recently there have been positive indications regarding the use of pyrethroids in integrated control measures targeting both tick and tsetse vectors, (Eisler, *et al.*, 2003). When properly optimized, this will clearly have advantages in the reduction in operational costs as well as empowering livestock owners and the private sector to be able to participate in these programmes. However, regardless of any programmes to be put in place, accurate epidemiological information about the diseases and their vectors is necessary in determining first, the impact of the diseases and secondly, the follow-up programmes after control measures have been applied. This in turn requires sensitive and specific methods that will demonstrate the presence or otherwise of these disease pathogens, both qualitatively and quantitatively.

In sub-Saharan Africa microscopy has been the most established and widely used method for diagnosing vector-borne pathogens. However, microscopic detection of these pathogens

has proved inadequate in addressing the issue of sensitivity and specificity. Indeed, although disease outbreaks associated with only one haemoparasite species can be important, it is often the case that several species and sometimes sub-species may concurrently occur in animals under field conditions. Such cases cannot be distinguished by the power of microscopy. For example, it is not possible to distinguish different *Theileria* species such as *T. parva* and *T. mutans* or the various *Trypanosoma brucei* sub-species such as *T.b. brucei* and *T.b. rhodesiense* by microscopy. Since control measures for the diseases caused by these organisms are expensive it is important to distinguish between the pathogenic species or sub-species in the animal or human host, from those that are non-pathogenic, in order to rationalize the control strategies.

Recently several DNA based molecular diagnostic tests have been developed that can address the gaps in which reliable microscopy may not be adequate. These molecular based techniques, though complex and expensive, have enabled many epidemiological questions, such as what pathogen species are found in animals in a particular place. For diagnostic purposes DNA-based techniques can resolve parasite identification at the species, sub-species and even strain level (McLaughlin, *et al.*, 1996). These molecular based methods such as the polymerase chain reaction, (PCR) amplification can also allow the impact of the diseases to be addressed with a high degree of accuracy. Further more, effectiveness of control measures such as chemotherapy can now be monitored more accurately.

The objective of this study was to make use of the molecular techniques to assess the impact of the vector borne diseases and determine the effect of drug intervention against natural infections of trypanosomes in cattle. Since many previous studies had relied on microscopy for screening of animals, (Uilenberg, 1998), its performance in detecting trypanosomes and tick-transmitted parasites was evaluated against molecular based methods. The data obtained in this study through molecular screening of a large number of cattle from owner managed village production system, have allowed for re-evaluation of such issues like distribution of both tick and tsetse transmitted parasites. The prevalence of pathogenic trypanosome species affecting cattle has also been re-evaluated. The effect of

both prophylactic and curative drugs in the control of animal trypanosomiasis was studied. The most vulnerable animal age-groups to trypanosome infection also formed part of the study.

Interaction between different pathogens within individual animals has been demonstrated in several studies. Heller-Haupt, *et al.*, (1983), demonstrated the interaction between *T. congolense* and the ecto-parasite, *Rhipicephalus appendiculatus* which is also the vector for *Theileria parva* in rabbits. Dossa, *et al.*, (1996) had also demonstrated the interaction in Boran cattle between the tick *Ambyoma variegatum*, *T. congolense* and *Babesia bigemina*. The interaction between *T. congolense* and *Haemonchus contortus* from an entirely different phylum has also been demonstrated in N'Dama cattle, (Kaufmann, *et al.*, 1992). All these have been experimental associations, but it is clear that in field situations several pathogen species may co-infect an animal resulting in increased severity of one disease as is in the case with the findings of Kaufmann *et al.*, (1992) or both diseases. Detailed analysis of pathogen interactions conducted from the same animals in this study is the subject of another study, (Tossas-Auguet, PhD Thesis, in preparation, University of Edinburgh). However, a more generalized description of distribution patterns of both tick and tsetse-transmitted parasites based on screening of cattle by both microscopy and molecular methods is discussed in this study.

#### **8.1.1 PCR amplification of *p104* gene of *T. parva* in cattle**

The detection of *Theileria parva* schizonts in cattle blood was achieved through PCR amplification of the *p104* gene. The use of either the internal or both external and internal primers in a nested PCR amplification had been compared by Odongo, (2004) where it was shown that the nested PCR amplification could detect more animals positive with *T. parva* than when the internal primers were used alone. However, in that study, no statistical analysis was done to compare the sensitivities of the two PCR based methods. In this study, the sensitivity of amplification with internal primers was found to be 5% while the specificity was 89%. This is an indication that the internal primer PCR technique can be used to detect *T. parva* but not other the species of *Theileria*, but it was not sensitive

enough to detect low parasitaemia. The predictive value probability for detecting p104 positives was 0.18 and for detecting p104 negatives was 0.93 while the Kappa value was 0.12. These are indications that there was no agreement between the two methods. These results also imply that the use of internal primers in PCR amplification of p104 gene in detection of *T. parva* is unsatisfactory when compared with the nested PCR amplification method. The analysis supported the observation by Odongo (2004), where it was shown that the sensitivity of PCR using internal primers alone was very low compared to the nested PCR amplification.

## **8.2 The tick-borne diseases in cattle from Tororo and Busia**

*Theileria mutans* was found to be more predominant at 42.2% (CI = 36.8 – 48.0), while the prevalence of *T. velifera* at 12.2%, (CI = 8.8 – 16.0) and *T. parva* 11.9% (CI = 8.6 – 16.0) were similar in the animals screened in Tororo. This is in conformity with previous findings in an endemic region of central Uganda, (Oura *et al.*, 2004), where the prevalence of *T. mutans* was found to be higher than *T. velifera* and *T. parva*. Similarly, in another study in Tanga and Iringa regions of Tanzania, (Ogden, *et al.*, 2003), it was observed that the high proportion of infection with *Theileria* parasites was due to higher prevalence of *T. mutans*. In another study in Kenya, (Watt, *et al.*, 1998), the piroplasms were seen in cattle blood but *T. parva* could not be demonstrated by PCR amplification, leading to the conclusion that other *Theileria* species rather than *T. parva* were being detected by microscopy. Combining the prevalence of *Theileria* species determined by molecular methods shows that the overall prevalence was 66.3% which was higher than the 48% previously reported in this region, (Anon, 1996).

The prevalence of *Theileria* species was higher in Tororo, 59% than in Busia, 38%,  $P < 0.0001$ . The prevalence of *Anaplasma* was also higher in Tororo 56% compared to 33% in Busia villages,  $p < 0.0001$ , as was determined by microscopy. There were no data of the prevalence of *Anaplasma*, *Babesia* or the *Rickettsial* species in the same samples obtained by molecular methods, despite the fact that microscopy detected high prevalence of *Anaplasma* at 58%. This was due to the fact that the reverse line blot assay did not detect

any animal positive with these parasites, despite the fact that even the seroprevalence of *Anaplasma* in a few selected animals in this study was also shown to be high at 74%, while *Babesia* was 67% (Magona, thesis, 2004 Glasgow University).

Generally, the prevalence of tick-borne parasites as screened by microscopy was higher in cattle from Tororo villages than in Busia. None of the animals screened in Tororo and Busia were positive for *Cowdria* and only 3 were positive for *Babesia*. However, it was not possible to determine which species they were infected with, since the cattle were only detected by microscopy.

While molecular methods such as PCR amplification and reverse line blot (RLB) assays provided the basis for specific species identification, in this study they lacked the ability to detect *Anaplasma* or *Babesia* parasites. In particular, although RLB provided the information on the prevalence of other *Thieleria* species such as *T. mutans* and *T. velifera* its sensitivity in detecting *T. parva* and other tick-borne parasites was questionable since it did not detect any animal positive. While the results are consistent with the observations by Odongo, (2004), that the sensitivity of RLB is too low for detecting *Anaplasma* and *Babesia*, this could also have been due to the fact that the samples were not stored in the manner that would have preserved these parasites and there is therefore need for further investigation to determine the applicability of this method in screening field samples. Moreover, the RLB method is more labour intensive and too costly for the screening of large number of samples and therefore may not be suitable as a diagnostic technique applicable for field evaluation of disease prevalence.

In conclusion, the results in this study have clearly shown that the prevalence of various tick - borne parasites infecting cattle in this area is much higher than previously estimated when molecular techniques are used. In particular, the prevalence of trypanosomiasis in this area of SE Uganda had been highly underestimated in the previous studies carried out by microscopy (Okuna, *et al.*, 1996; Magona and Mayende 2002). However, the use of microscopy still provided the best option for estimating the prevalence of tick-borne



parasites other than *Theileria* species. Furthermore, many epidemiological studies will still require the use of microscopes for morphological characterization of disease pathogens affecting humans and livestock. This can only be achieved by microscopic examination of the specimens.

### 8.3 Trypanosomiasis

The results obtained in this study showed that the diagnostic performance of microscopy in detecting animals infected with trypanosome species was too low compared to PCR amplification. The kappa values computed for both individual species or for all species were below the 0.4 cut off point for the two methods to have been considered to be in agreement, (Fleiss, 1981). This is an indication that while reliability on microscopy detecting animals with infection is unsatisfactory, its reliability in detecting animals without infection is good. However, microscopy had good specificity (> 90%), for the detection of all the three species of trypanosomes.

It is interesting to note that microscopy was able to detect 10 animals (1.2%) which were not detected by PCR amplification. Since the specificity of microscopy in detecting correct species was being tested against PCR amplification as the standard, a more likely explanation for this observation is that these could have been other species, since PCR amplification detected 75 animals (11.8%) as having *T. brucei* infection that were not detected by microscopy. These results were in agreement with the previous findings where it was shown that out of the 402 animals sampled from a group of small ruminants in Busia Kenya, microscopy only detected 5 cases of trypanosomiasis of which only one was *T. congolense* infection while PCR amplification detected 21 cases of *T. congolense* infections, (Ngayo, *et al.*, 2005). It has also been shown that whereas microscopy was able to reveal 42% cases of positive *T. vivax* infection, PCR amplification revealed 75% in the same animals (Masake, *et al.*, 1997). In their study in Uganda, Clausen *et al.*, (1999), also found that microscopy showed the prevalence of trypanosomes to be 18.9% against 34.8% by PCR amplification. Picozzi and others, (2002), also found that microscopy detected 13 cases of *T. brucei* in cattle blood against 47 detected by PCR amplification using FTA filter

cards. More recently, Ngayo, *et al.*, (2005), found that microscopy could only detect 3 animals infected with *T. brucei* in small ruminants against 20 that were detected by PCR amplification.

The proportion of animals that were detected in this study with the three species of trypanosomes by PCR amplification but missed by microscopy was 14%. This is a clear indication that the use of microscopy in diagnosing animal trypanosomiasis underestimates the real disease situation and therefore unsuitable for quantifying the disease burden in cattle. In conclusion, the results obtained in this study have provided important statistical comparisons that can be used in decision making with regards to the data collected using microscopy in detecting trypanosomes in animals for epidemiological studies.

### **8.3.1 The prevalence of *T. brucei* in cattle**

The initial prevalence of *T. brucei* in individual villages of Tororo and Busia districts as determined by PCR amplification was quite varied, ranging from 3.8% in Ojilai to 13.8% in Magoje in Tororo and from 7.5% in Buyimini, to 13.8% in both Kubo and Nanjeho. The overall baseline prevalence in cattle from Tororo villages was found to be 7.5% while in Busia the prevalence was 16%. The overall initial prevalence was much higher than previously recorded in cattle in this area, (Ford, 1971; Magona and Mayende, 2002), when microscopy was used to screen the animals for infection. In both districts the prevalence dropped to below the baseline levels in the first five months and there was no significant difference between isometamidium chloride - treated and untreated animals. However, there was a major upsurge of prevalence in the treated cattle in the sixth month following treatment that resulted in a significant increase in the mean prevalence above the untreated group,  $p < 0.01$ .

However, a comparison of the incidence of infections with *T. brucei* in both isometamidium treated and untreated cattle from Busia and Tororo districts by using survival analysis showed that there was no significant difference in infection rate,  $p = 0.074$ , with *T. brucei* between treated and untreated cattle in Tororo district, although only

3% of the treated animals survived without PCR detectable infections up to 161 days compared to 49% in the untreated group. Similarly, there was no significant difference between the two groups in Busia district,  $p = 0.118$ , with 46% of the treated animals surviving without PCR detectable infection up to 161 days compared to 63% in the untreated animals.

Finally, when all the data from both districts were pooled together the result showed no significant difference between the two groups, Figure 49, ( $p = 0.986$ ), with 22% of the treated animals surviving up to 161 days without PCR detectable infection, compared to 54% in the untreated group. Generally, more untreated animals survived up to 161 days without PCR detectable trypanosomes than the treated ones and treatment of cattle appeared to have a negative rather than a protective effect in the control of infection with *T. brucei* in cattle. These observations are contrary to what had been found previously that isometamidium chloride treated cattle had higher proportion of cattle free of *T. brucei* re-infection than the untreated ones, (Magona, *et al.*, 2004). It is not clear what this observation could be attributed to. However, a more likely explanation is that it could have been due to the fact that the animals lost the acquired resistance to this species of trypanosome following treatment. The other possibility is that of drug resistance, although in a previous study, Enyaru and others, (1998), concluded from their *in vivo* experiments in mice with a strain of *T. brucei rhodesiense* that drug resistant trypanosomes are probably not circulating in SE Uganda.

The cumulative proportion of re-infection of cattle with *T. brucei* following treatment reached 32% in the group of animals that had trypanosomes before treatment while the proportion reached 36% in the group that had no trypanosomes prior to treatment. These observations suggest that the infection rate was the same in treated and untreated animals and could be attributed to new transmission, rather than breakthrough due to treatment failure. However, the results also imply that the animals could only be protected for four months after which the animals become vulnerable to new and higher infections.

Using a simplified model that considered the relationship between the prevalence of *T. brucei* infection and age of the animals it was observed that the prevalence had significant difference association with age,  $p = 0.0001$ , as it showed that *T. brucei* could be detected in more animals that were 13 months old and above than in calves below 12 months old. The prevalence was highest in adult animals over 24 months old, suggesting that *T. brucei* infections increased with the age of the animals. The mean proportion of infected cattle was 10.14% for adults compared to 6.37% in calves and the adults were less likely to be detected with *T. brucei* infection than adults, relative risk (RR) = 37% in calves vs. 63% in adults. There are however no literature describing similar finding and the possible explanation for this observation is that animals grow with *T. brucei* since it is known to cause sub-clinical disease in cattle, (Morrison, *et al.*, 1983), and may not show any clinical signs that would warrant intervention by the farmer.

The results in Busia were consistent with previous studies where *T. brucei* was predominant in areas endemic for sleeping sickness, (Magona, *et al.*, 1999). Although the samples from Busia animals were not screened for *T. b. rhodesiense* SRA gene, it is possible that some of the *T. brucei* positives could be human infective, like the cases in Tororo. It is interesting to note that the ratio of *T. b. rhodesiense* among the group of cattle detected with *T. brucei* infection remained nearly the same as the baseline samples, at 1:4 at different villages, time of the year and even six months after prophylactic drug intervention.

### **8.3.2 The prevalence of *T. congolense* in cattle**

The initial prevalence of *T. congolense* in the individual villages of Tororo as determined by PCR amplification ranged from 3.8% in Bunghaji to as high as 20% in Magoje. In the villages of Busia, the initial prevalence ranged from 8.8% in Buyimini 30.0% in Sitengo. The overall baseline prevalence in cattle from Tororo villages was found to be 13% while in Busia the prevalence was 21%. The overall initial prevalence was much higher than previously recorded in cattle in this area, (Ford, 1971; Magona and Mayende, 2002), when microscopy was used. In both districts the prevalence dropped to below the baseline levels in the first three months and there was no significant difference between isometamidium

chloride - treated and untreated animals. However, in the fourth month the prevalence rose higher in the untreated animals from Tororo cattle than the treated ones, suggesting that treated animals had some protection. This was exactly the opposite in the cattle from Busia where the prevalence rose higher in the treated animals than the untreated at the same time point. This was likely to have been due to the fact that the initial prevalence was higher in cattle from Busia than Tororo. The incidence of new *T. congolense* infections in Tororo continued to rise up to the fourth month when it reached 29, the highest number of new cases. In Busia, the incidence continued to rise up to 43 new cases in the same time point. These results further suggest that treatment of cattle provided protection against infection in Tororo where the initial prevalence was low but not in Busia where the initial prevalence of *T. congolense* was high.

When the comparative analysis was done of the rate of infection with *T. congolense* in both isometamidium treated and untreated cattle from Tororo and Busia districts, the results showed variations in the two districts. While there was significant difference in prevalence between treated and untreated animals in Tororo,  $p = 0.0001$ , this was not the case in Busia where the prevalence between treated and untreated animals was not different,  $p = 0.808$ . In Tororo, the proportion of animals that survived without PCR detectable infection up to 161 days was 96% in the treated and 88% in the untreated groups respectively, while the proportion of the animals that survived infection for the same period in Busia was the same, 74% in both groups. These results were confirmed when the analysis was done on the data from Busia cattle, to test the null hypothesis that the mean prevalence in untreated is equal to that of treated animals, Test statistic  $t = -0.77$  on 50 d.f.  $p = 0.447$ , indicating that there was no significant difference in prevalence between treated and untreated animals. The results from Busia cattle were consistent with studies where drug resistance has been observed, (Afewerk, *et al.*, 2000, Rowlands, *et al.*, 2001).

However, the overall effect of treatment was confirmed when all the data from both districts were pooled together. The result showed that there was a significant difference between the two groups,  $p = 0.0002$ , although 89% of the treated animals survived up to

161 days without infection compared to 83% in the untreated group. The cumulative proportion of re-infection of cattle with *T. congolense* following treatment reached 21% in the group of animals that had trypanosomes before treatment while the proportion reached 16% in the group that had no trypanosomes prior to treatment. Even though the proportion of the animals that survived without infection was not so different, the analysis strongly suggests that the infection rate was low in both groups irrespective of treatment. This does not give a clear indication of whether or not the treatment had any effect on the infection rate of *T. congolense*.

The proportions of various *T. congolense* types in cattle from the villages of Tororo and Busia districts as was determined from PCR amplification is showed that Savannah type was predominant at 51% in Tororo and 63% in Busia. This was followed by the Forest type at 32% to 42%, while and 7% were Tsavo type. Only 2% had mixed infections of the Savannah and Forest types. Overall, the proportion of various *T. congolense* types in cattle from the villages of both districts, as determined by PCR amplification, showed that the Savannah type was more predominant at 57% while the Forest type was 37% as the Kilifi type made up less 1% and 6% were the Tsavo type. This was consistent with the geographical locations of the study villages which were mostly situated in the Savannah grassland with some forest cover near by. However, the predominance of the Savannah type of *T. congolense* would not be consistent with its higher pathogenicity previously shown by Sidibe, *et al.*, (2002) as it would mean high mortality rates which has not been documented in this area.

As with *T. brucei*, it was observed that there was no interaction between the prevalence of *T. congolense* in the animals and their sex,  $p = 0.9063$ , despite some variation in the proportions, (10% males compared to 17% females), as the animals grow older. However, unlike the observation with *T. brucei*, the analysis showed that the animals in the 1-24 months age group were more likely to have infection with *T. congolense* than the adults,  $p < 0.0001$ , as detected by PCR amplification. The relative risk (RR) of *T. congolense* detectable by PCR in young animals was 66.63%, compared to the adults, thus younger



animals were 33% more likely to have *T. congolense* infection as detected by PCR amplification than the adults. The possible explanation for this observation is that since the infections with *T. congolense* present observable clinical signs, the affected animals receive remedial attention right from an early age, thus reducing the proportion of adult animals that had detectable trypanosomes. Since the general practice in the study area is to keep the calves tethered in or near the homestead, these findings could be interpreted to mean that the vector has become more peri-domestic. These results appeared to contradict what had been found in southwest Ethiopia, where it was shown that the mean monthly prevalence in cattle increased significantly with age, (Rowlands, *et al.*, 1993; 2001).

### **8.3.3 The prevalence of *T. vivax* in cattle**

The initial prevalence in the villages of Tororo ranged from 6.3% in Bunghaji to 36.3% in Magoje. In the villages of Busia, the initial prevalence ranged from 5% in Kubo to 26.3% in Sitengo. In Tororo, the initial prevalence was three times, 18.6% vs. 6.3% higher in the treatment animals than those villages that were not to be given treatment the prevalence dropped in both groups up to the third month, however, the prevalence rose significantly higher in the untreated cattle, 26.2% compared to 1.3% ,  $p < 0.001$ , in the fourth month. In Busia the initial prevalence in the treatment group was lower than in the non- treatment group of cattle, 6.3% vs. 20.6%,  $p < 0.001$ . Subsequently, there was no difference in prevalence in the two groups up to the 5<sup>th</sup> month when the prevalence remained at 1.8% in the treated cattle but rose to 12.7%,  $p < 0.001$ , in the untreated animals. After treatment the incidence of new infections of *T. vivax* in cattle from Tororo villages reached 57 cases, the highest cumulative incidence in the fourth month while in Busia villages, the incidence reached 33 new cases in the sixth month.

When the data from the two districts was combined the results showed that the initial prevalence of treatment cattle was not different from the no treatment ones, 15.0% vs. 17.0%,  $p = 0.5$ . However, in the following month the prevalence was slightly lower in the treated cattle than the untreated, 0.3% vs. 2.8,  $p = 0.04$ . Subsequently there was no difference in the prevalence between the two groups of animals. This was confirmed when

the analysis was done to test the null hypothesis that the mean of prevalence in untreated is equal to the mean of treated and the result showed a test statistic,  $t = -0.50$  on 18 d.f.,  $p = 0.623$ , indicating that there was no significant difference in prevalence between treated and untreated animals.

When the comparative analysis was done of the rate of infection with *T. vivax* in both isometamidium treated and untreated cattle from Busia and Tororo districts there were some variations in the two districts. In Tororo the difference in infection between treated and untreated animals was not significant, Figure 71, ( $p = 0.12$ ), while the proportion of the animals that survived without infection up to 161 days was 69% in the treated and 78% in the untreated groups respectively, suggesting that treatment did not confer protection to the animals. However, in Busia there was significant difference in survival between treated and untreated cattle,  $p = 0.0002$ , with 86% of the animals surviving without infection up to 161 days in the treated group compared to 66% in the untreated group, implying that treatment conferred protection against infection.

These results suggest that *T. vivax* infection rate could be controlled with drug treatment in Busia where the prevalence was lower but not in Tororo where it was high. When all the data from both districts were pooled together the result showed that there was significant difference in survival without infection between the two groups,  $p = 0.0002$  although the proportion of the animals which survived was not so different, 74% in the treated and 70% in the untreated group. These results further imply that treatment of cattle had an overall protective effect in the control of infection with *T. vivax* in cattle for up to six months.

The cumulative proportion of re-infection of cattle with *T. vivax* following treatment reached 36% in the group of animals that had trypanosomes prior to treatment while the proportion reached 40% in the group that had no trypanosomes, indicating that the infections were likely to be new rather than breakthroughs. Generally, the proportion of animals with infection was higher in the group that had trypanosomes prior to treatment than those that did not have infection.

As with *T. brucei* and *T. congolense*, it was observed that there was no interaction between the prevalence of *T. vivax* and the sex of the animals  $p = 0.1531$ , despite some variation in the proportions, 15% males compared to 25% females, as the age of the animals increased. However, it was observed that younger animals were more likely to have *T. vivax* infection than the adults,  $p = 0.0006$ , as detected by PCR amplification. The relative risk (RR) of *T. vivax* detection by PCR in calves compared with adults was computed to be 71.27%, thus young animals were approximately 29% more likely to be found with *T. vivax* infection than the adults as detected by PCR amplification. The possible explanation for this observation is like with *T. congolense*, this species which is highly pathogenic, manifests in clear clinical signs that necessitate mitigation as the animals grow. As had been discussed, the possible vector adaptation to the peri-domestic environment could also play a part in the increased disease transmission. These results are also contrary to what had been found by Rowlands, *et al.*, (1993; 2001), where the prevalence of *T. vivax* increased with age of the animals. It is interesting that in their study, (Rowlands, *et al.*, 2001) they found that the *T. vivax* infecting the animals were less pathogenic and therefore may not have exhibited clear clinical signs.

#### **8.3.4 The prevalence of any trypanosome species in cattle**

Overall, the prevalence of trypanosomes was higher in Busia than Tororo cattle. The initial prevalence in Tororo in the treatment group of cattle was significantly higher than in the no treatment animals, 42.2% vs. 21.5%,  $p = 0.0001$ . After treatment, there was no significant difference in the subsequent months up to the fourth month when the prevalence in the treated cattle was lower than in the untreated animals, 14.7% vs. 34.6%,  $p < 0.0001$ . However, in the 6<sup>th</sup> month, the prevalence was significantly higher in the treated cattle than the untreated ones, 56.3% compared to 16.4%,  $p < 0.0001$ . This was largely due to the upsurge of *T. brucei* in the treated animals in the sixth month. In Busia there was no significant difference in initial prevalence in the treatment and no treatment groups, 36.3% vs. 41.9%,  $p = 0.76$ . However, in the five months following treatment, the prevalence was significantly lower in the treated cattle than the untreated ones, 3.4%, 8.8% and 8.8% vs.

11.1%, 22.9 and 26.3% respectively,  $p < 0.05$ . As in Tororo the prevalence in the treated cattle rose significantly higher than in the untreated animals, 40.6% compared to 22.9%,  $p = 0.01$ , largely due to the upsurge of *T. brucei*.

When the data from the two districts was combined the results showed that the initial prevalence of any trypanosome in the treatment cattle was not different from the no treatment ones, 39.3% vs. 31.8%,  $p = 0.54$ . However, in the following two months the prevalence was reduced in the treated cattle than the untreated, 1.7% and 6.3% vs. 6.0 and 15.9%,  $p < 0.03$ , indicating that mass treatment was able to reduce the prevalence of all trypanosome infections. This was however shortlived as there was no difference in the prevalence between the two groups of animals from the third month up to the sixth month, when the prevalence in the treated cattle rose significantly higher than the untreated animals, 49.4% vs. 19.5%,  $p < 0.0001$ . This overall difference was largely due to the upsurge in the prevalence of *T. brucei* in treated animals in both districts. Moreover, when a test of the null hypothesis that the mean of mean prevalence with untreated was equal to that of treated cattle, was done, it was observed that a Test statistic  $t = -0.35$  on 39.46 d.f. with probability = 0.731, indicating that there were no significant differences in the mean prevalence between treated and untreated animals, leading to the conclusion that treatment of cattle with isometamidium chloride did not confer protection to animals under trypanosome infection in areas where the prevalence of trypanosomes is high. These results are consistent with previous observations that the periodic protection of cattle afforded by isometamidium chloride was inversely related to the prevailing challenge, (Stevenson, *et al.*, 1995).

When the data for all trypanosome species infection was combined and the comparative analysis done in both isometamidium treated and untreated cattle from Busia and Tororo districts it was shown that there was significant statistical difference in survival of animals without detectable trypanosome infections,  $p = 0.0234$ , between treated and untreated animals, despite the fact that the proportion of the animals that survived without infection up to six months was 74% in both groups. The cumulative proportion of re-infection of

cattle with any trypanosome species following treatment reached 30% in both groups of animals that had and did not have trypanosomes before treatment in six months. The results also show that there were detectable trypanosomes in both group as early as day 21, (<1%) but reached above 10% three months after treatment. These results suggest that the infections could have been both breakthroughs due to treatment failure and new infections due to lack of protection by the drug. However, the results also imply that treatment of the animals could only confer protection against infection with any species of trypanosomes for three months.

Except in the first month when there was a significant reduction in prevalence in treated animals, the overall prevalence as determined by microscopy was not different in treated and untreated cattle from both districts, suggesting that mass treatment had no effect in the control of trypanosomiasis. This observation is important since many previous studies using microscopy, including the more recent one, (Magona, *et al.*, 2004), have shown a protective effect for up to three months following treatment with isometamidium chloride. However, in Magona, *et al.*'s study, the area had a suppressed vector population, meaning that the prevalence of trypanosomes could have also been low. However, there is need to conduct further studies that would include the vector activities and quantitative measurements of trypanosome challenge in this area. It is important to note that there were no data regarding tsetse density and trypanosome transmission dynamics during the period of this study. This could provide some insight as to whether there was clear drug resistance in the study area or if it was simply due to high transmission rate that could have overwhelmed the effect of treatment as was observed with *T. vivax* in Tororo district. In conclusion, these results suggested that treatment of cattle with isometamidium chloride did not confer any protection against infections by any of the three species of trypanosomes, as determined by either PCR amplification or microscopic examination. Instead, the upsurge of *T. brucei* prevalence in the treated animals, as was found with PCR amplification, appears to complicate the estimation of the true effect of the drug.

The distribution of the three pathogenic trypanosome species in various animal age-groups showed that *T. congolense* and *T. vivax* infections were more likely to be detected in young animals by PCR than *T. brucei*. However, as the animals grew the distribution of the three trypanosome species was similar at 34% for *T. brucei*, 38% for *T. congolense* and 35% for *T. vivax*. This equal distribution gave way to *T. brucei* predominance as the animals mature: *T. brucei* at 55%, followed by *T. vivax* at 40% and *T. congolense* at 38%.

#### **8.4 Effect of treatment of cattle with isometamidium chloride on anaemia**

Anaemia has been found to be the key pathological indicator of trypanosomiasis, (Murray and Dexter, 1988). Anaemia was assessed by the measurement of haemoglobin concentration in all the animals. The results of the monthly mean haemoglobin concentration in treated and untreated cattle from Tororo and Busia, was not significantly different,  $p > 0.05$ . These results imply that mass treatment with isometamidium chloride does not have any significant effect on the state of anaemia caused by trypanosomiasis. However, it should be noted that anaemia could have been due to other pathogens such as *Anaplasma*, affecting the animals which were not targeted by treatment. These findings are consistent with the results of a study carried out by Efewerk, *et al.*, (2000), in which they observed that the mean packed cell volume (PCV) of East African zebu cattle treated with isometamidium chloride did not differ from those not given treatment for up to three months. In their study, the animals were also kept under traditional husbandry and therefore there was a possibility that the animals had infections with other anaemia inducing pathogens.

#### **8.5 Effects of treatment of cattle with diminazene on the prevalence of trypanosomes in cattle**

Analyses were performed using a linear mixed model to test whether there was any effect on the change in prevalence of trypanosomes 28 days after treatment with diminazene, as determined by both microscopic examination and PCR amplification. The proportion of animals that were detected with trypanosomes by microscopy 28 days after treatment in the group of animals that had infection prior to treatment was about 7% while those which did



not have infection prior to treatment was 4%. The proportion of animals that had detectable trypanosomes in the untreated animals was more than that of the treated animals, 4.32% compared to 2.86%. The calculated relative risk indicated that there was 33.8% more risk of positive diagnosis in untreated animals, regardless of whether they had been diagnosed with trypanosomes in the previous month or not. However, the analysis indicated that there was no interaction between diminazene treatment and the detection of trypanosomes after 28 days by microscopy,  $p = 0.1103$ , indicating that treatment with diminazene does not confer protection against infection beyond 28 days.

The lack of significant interactions when microscopy data on previous month – diminazene treatment,  $p = 0.1103$  and PCR data on previous month – diminazene treatment,  $p = 0.1367$ , suggests that both a clearance and to a much lesser extent, a protective effect occurred, and was visible 28 days after administering the drug. Therefore, the hypothesis that diminazene clears, but does not prevent infection or re-infection of cattle with trypanosomes 28 days after its administration does not hold true. If the interaction had been significant, it would have been expected that the proportion of positive animals remaining positive the following month after treatment would be lower while the proportion of negative animals becoming positive would have not varied depending on whether diminazene was given or not. The proportion of positive animals on the following month after treatment was higher in animals which were diagnosed with the parasites by PCR on the previous month, 19.15%, compared to those which were diagnosed negative, 13%. The same was observed with microscopic examination, 7% compared with 4%, suggesting that trypanosome infections are unlikely to last for less than 28 days at detectable levels using microscopy and particularly, PCR amplification

The proportion of the previously infected animals that were detected with trypanosomes by PCR amplification 28 days after treatment was 19% compared to 13% in animals which previously had no infection. Overall, there was no major difference between proportion of trypanosome positive observations in treated animals, 11.41%, and the untreated animals, 14.07%, with PCR amplification. The relative risk indicated that there was 18.93% more

risk of positive diagnosis in untreated animals regardless of whether they had been previously diagnosed with trypanosomes or not. This was about half of what was found with microscopy data, further indicating that there is more likelihood of detecting trypanosomes in cattle even after treatment by PCR than microscopy. Like with microscopy however, the analysis indicated that there was no interaction between diminazene treatment of cattle and trypanosome detection after 28 days,  $p = 0.1367$ .

Trypanosome infected cattle treated with diminazene had 75% and 49% less relative risk of remaining detectable by microscopy and PCR respectively, compared to those which did not receive the drug. However, previously negative animals had 44% and 14% less relative chances of becoming detectable by microscopy and PCR respectively. Since the effects of diminazene appeared to be far more obvious when considering microscopy data, it is reasonable to conclude that diminazene lowers the parasitemia in the animals below the minimum detectable threshold of microscopy rather than achieving complete clearance of the infection. Also, it is interesting to note that not all animals which received treatment against trypanosomiasis cleared the infection detectable by either technique and thus caution should be placed when deciding to use this drug to reduce the transmission of the parasite. However, it is evident that whereas diminazene treatment can be used to contain clinical disease it appears to leave residual infections in animals that act as healthy carriers. This might explain why in previous reports it was stated that diminazene could be used to clear trypanosome reservoir infection in animals for only two weeks, (Magona, *et al.*, 2004). It has also been shown that when diminazene is used to treat animals in areas where tsetse control is in practice the drug significantly reduces the prevalence of trypanosomes, (Fox, *et al.*, 1991; Leak, *et al.*, 1995). Rowlands, *et al.*, (1995), had also shown that cattle remained productive where tsetse control and diminazene treatment are used concurrently.

Equally interesting was the fact that infected cattle had 75% less relative risk of trypanosome detection by microscopy, when compared with 44% in those which did not receive the drug while previously negative. The observation was more evident with PCR amplification method, 49% less relative risk in infected cattle compared to 14% for those

which received drug while previously negative. These observations seem to imply that diminazene treatment appeared to have some residual effect on trypanosome infection. Since the effects of diminazene appears to be far more obvious when considering microscopy data which has been shown to have poor diagnostic sensitivity, it is reasonable to conclude that diminazene lowers parasitaemia rather than achieving complete clearance of the infection as had been hypothesised. It is worth noting that not all animals which received diminazene treatment had the infection cleared as determined by either technique indicating that treatment of cattle with this drug cannot be relied upon to reduce the transmission of the parasite.

Overall, the proportion of positive observations was higher in non treated animals than in treated, regardless of whether they had been diagnosed with trypanosomes in the previous month or not. When using the data from PCR amplification the proportion of animals without previous treatment that had detectable trypanosomes was 14.07% compared to 11.41% in the animals that had previous treatment. The relative risk showed that 19% of the animals without previous treatment were more likely to be diagnosed with trypanosomes. However, when the data from microscopic examination was used the proportion of the animals that had no previous treatment that had detectable trypanosomes was 4% compared to 3% in the animals that had previous treatment. The relative risk showed that there was 33.8% more risk of detecting trypanosomes in the non-treated. These differences in the relative risks between the PCR and microscopy data explain why the effect of diminazene using PCR data is only marginally significant ( $p = 0.0443$ ), whilst when considering microscopy data it is found to be highly significant ( $p = 0.003$ ).

## 8.6 Conclusions

The overall objective of this study was to understand the epidemiology of the vector-transmitted diseases in cattle reared under mixed farming management system in the villages of SE Uganda. In order to achieve this, two of the most commonly used technologies, microscopy and molecular, for pathogen detection and species characterization were evaluated for their sensitivity and specificity and utilized in detecting

the parasites in the blood of cattle. The results obtained in this study have confirmed that the sensitivity of microscopy in detecting all the three trypanosomes species, in relation to PCR amplification is unsatisfactory. Furthermore, the analysis has resulted in important statistical comparisons that can be used in decision making with regards to the data collected using microscopy in detecting trypanosomes in animals for epidemiological studies.

The study also aimed at making use of the available molecular markers and methodologies to characterize the two main groups of vector-borne parasites – tick and tsetse transmitted parasites circulating in cattle in Busia and Tororo Districts of south east Uganda and to quantify the impact of trypanosome prevalence in the area. The study has shown that the prevalence of all trypanosome species is much higher when determined by PCR amplification than microscopy. More importantly, the study has shown that the prevalence of tsetse transmitted trypanosome species in cattle is far much higher than previously documented in this region. The results have further confirmed that some of the animals infected with *T. brucei* actually had the human infective *T.b. rhodesiense*.

The study also sought to assess the impact of drug intervention on the prevalence of trypanosomiasis in indigenous cattle based on molecular screening method of trypanosome detection in cattle. Overall, the results showed that significant control in infection rate following drug treatment occurred but only in the areas where the prevalence was low, for example, in Tororo where the prevalence of *T. congolense* was low or in Busia where the prevalence of *T. vivax* was low. This seems to suggest that effectiveness of prophylactic treatment of cattle against animal trypanosomiasis could be linked to the disease transmission rate and can be achieved where trypanosome challenge is low (Fox, *et al.*, 1991). While some protective effect of isometamidium chloride treatment of cattle on the prevalence of the three trypanosome species in both districts was apparent as determined by PCR amplification, this was not evident with microscopy. The study also showed that treatment had a negative effect on the control of *T. brucei* infections in cattle six months after treatment. The reason for this peculiar observation, in both districts, is unclear and

needs to be investigated further. The effects of diminazene treatment appeared to be far more obvious when considering microscopy data which has poor diagnostic sensitivity. This is an indication that diminazene lowers parasitaemia rather than achieving complete clearance of the infection. The implication of this observation is that treatment of cattle with this drug cannot be relied upon to reduce the transmission of the disease.

The analysis of the data obtained by PCR amplification showed no relationship between drug treatment and anaemia as the results of the monthly mean haemoglobin concentration in cattle from Tororo and Busia, indicate that there was no significant difference between treated and untreated animals for the first five months. Finally, the study also revealed that younger animals had higher prevalence of *T. congolense* and *T. vivax* than adults while the *T. brucei* was more prevalent in adult animals. The study has added important information that can be used in rationalizing disease control strategies in endemic areas in general and SE Uganda in particular.

## CHAPTER NINE

### 9. REFERENCES

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